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Diversidad y aspectos microevolutivos en cosimbiontes liquénicos

Microevolutive aspects and diversity in lichen co-symbionts

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Universidad Complutense de Madrid, Facultad de Farmacia

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“Nada en biología tiene sentido sino es bajo la luz de la evolución”

Theodosius Dobzhansky

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RESUMEN

Diversidad y aspectos microevolutivos en cosimbiontes de líquenes

Introducción Los líquenes son paradigma de estudios de simbiosis y resultan interesantes como objeto de estudio ecológico, biogeográfico y también reproductivo por sus múltiples modos de reproducción y sistemas de emparejamiento. El estudio de la microevolución, evolución a nivel de especie o inferior, requiere la delimitación precisa de las especies, que puede ser muy complicada debido a la presencia de especies crípticas, morfológicamente indistinguibles. Aunque hay múltiples conceptos de especie a nivel teórico, el más aceptado es el Concepto General de Linaje, la taxonomía integrativa trata de satisfacer los distintitos criterios de especie, al integrar múltiples líneas de evidencia para delimitar especies. Los análisis basados en coalescencia son muy útiles para descubrir diversidad oculta en especies polifiléticas. La diversidad oculta se ha podido hallar en los fotobiontes intratalinos de diversas especies al utilizar los marcadores moleculares apropiados. Esta variabilidad de fotobionte confiere mejor adaptación al medio ambiente y está influenciada por el tipo de reproducción del líquen. El cultivo axénico de los simbiontes liquénicos puede aplicarse a la obtención de tintes y principios activos para las industrias textil y farmacéutica, a la secuenciación genómica o a conocer las estrategias reproductivas de los líquenes. Las repeticiones de secuencias cortas (microsatélites) son regiones genómicas muy variables y los marcadores moleculares diseñados para amplificar estas regiones se utilizan para estudios de genética de poblaciones y de ecología molecular. Para estar seguros de la especificidad simbiótica de los marcadores moleculares es conveniente obtenerlos a partir de cultivos puros.

Objetivos El objetivo general de esta tesis ha sido analizar la diversidad presente en las poblaciones de líquenes y la implicación de la diversidad genética en la especiación en líquenes.

- Evaluación de la diversidad biológica real en el complejo de especies *Punctelia rudecta*, y comprobando si es un taxón monofilético o polifilético y elucidando la especiación críptica.

- Evaluación de la diversidad genética del fotobionte *Trebouxia decolorans* en el seno del talo liquénico.

- Cultivar en condiciones aposimbióticas el micobionte de *Parmelina* para conocer mejor algunos aspectos de su desarrollo ontogenético y su reproducción.

- Desarrollar marcadores genéticos de microsatélites de alta resolución específicos del micobionte de *Parmelina carporrhizans* para realizar estudios poblacionales.

- Estudiar la diversidad genética y la estructura poblacional de *Parmelina carporrhizans* y su distribución espacial. Comparar nuestros resultados con estudios previos en líquenes con otros modos reproductivos.

Síntesis Los cinco capítulos incluidos en esta memoria de tesis se corresponden con artículos científicos, cuatro de ellos han sido publicados en revistas científicas indexadas por el *Journal Citation Reports*, el capítulo 3 ha sido enviado a la revista *The Lichenologist* y está en proceso de revisión. De los artículos publicados, tres de ellos están el primer cuartil de sus respectivas áreas temáticas (JCR).

Hemos estudiado la diversidad biológica de *Punctelia rudecta* sensu lato, con una distribución disyunta similar a la Rand-flora pero además con presencia en Norte América. Para este estudio hemos seleccionamos tres marcadores moleculares basados en secuencias (ITS, RPB1 y LSU) que han demostrado ser útiles para distinguir especies. Hemos analizado la información obtenida de estos marcadores moleculares con métodos estadísticos de delimitación de especies, algunos de ellos basados en coalescencia. Además para mejorar la delimitación de especies hemos implementado un enfoque de taxonomía integrativa estudiando la morfología, la química, la ecología, y la geografía. Como resultados principales hemos constatado la polifilia críptica del taxón *P. rudecta s. lat.*, en el que hemos descrito y tipificado una nueva especie endémica de Canarias *P. guanchica.* Además hemos generado una filogenia del género *Punctelia*, que lejos de estar completa, es la mejor hasta la fecha comprendiendo casi una tercera parte de las especies. En esta filogenia hemos detectado cinco clados mayores con soporte estadístico que se caracterizan por las sustancias medulares que producen.

También hemos estudiado la variabilidad intratalina del fotobionte *Trebouxia decolorans* en las especies *Anaptychia ciliaris* y *Xanthoria parietina*. Esta investigación se ha desarrollado utilizando marcadores moleculares de alta resolución del tipo microsatélites (SSR), con ellos hemos genotipado el fotobionte en distintas porciones del talo liquénico y comprobado si la identidad del fotobionte era o no la misma en todo el talo. Nuestros resultados han mostrado múltiples fotobiontes en el 60% de los talos

de *A. ciliaris* y el 90% de los talos de *X. parietina*. Además en *A. ciliaris* hallamos de forma frecuente alelos múltiples en el genotipado de porciones de talo. Mientras que en el fotobionte de *X. parietina* hallamos mayor riqueza alélica y alelos menos relacionados. Discutimos que la adquisición de linajes distintos de fotobionte en *A. ciliaris* y *X. parietina* se debe a la generación de diversidad *de novo*. Aunque en el caso de *X. parietina* la gran riqueza alélica y menor relación entre alelos sugiere que además nuevos linajes de fotobiontes se adquieren desde el medio externo, bien al inicio del proceso de liquenización, bien a lo largo de la vida del talo.

En esta tesis hemos desarrollado cultivos aposimbióticos del micobionte de los líquenes *Parmelina carporrhizans* y *Parmelina quercina*. Con el objetivo de comparar el desarrollo ontogenético y algunos aspectos de la biología reproductora de estas especies. En nuestro trabajo hemos medido diversos parámetros, como son la producción de esporas, número de esporas eyectadas y porcentaje de germinación distinguiendo entre especies y entre apotecios grandes y apotecios pequeños. Aplicando la técnica de la placa invertida, hemos obtenido gran cantidad de esporas eyectadas por parte de ambas especies, entre cientos y miles por cada apotecio. *P. quercina* eyectó más esporas que *P. carporrhizans* y los apotecios grandes más que los pequeños. La germinación fue muy diferente entre estas dos especies, mientras que en *P. carporrhizans* germinaron el 90% de las esporas apenas el 0.5% de las esporas de *P. quercina* germinaron. Más aún las esporas germinadas de *P. quercina* rara vez llegaban a formar un micelio y observamos degeneración de las hifas a los pocos días de la germinación. No hallamos diferencias en la germinación de los apotecios grandes y pequeños. Ambas especies produjeron sustancias fenólicas que pigmentaron las colonias.

Tras cultivar satisfactoriamente *P. carporrhizans* y obtener biomasa suficiente, secuenciamos el genoma de la especie usando secuenciación de última generación, Illumina. A partir de este genoma, con el programa msatcommander diseñamos marcadores genéticos que amplificaran regiones de DNA que contengan entre 15 y 20 repeticiones de secuencias simples (SSR). Estos marcadores genéticos fueron ensayados para seleccionar aquellos que eran más variables y amplificaban en la mayoría de muestras, con el objetivo de ser utilizados en estudios poblacionales.

El estudio poblacional utilizando microsatélites y genes del locus MAT mostró una alta variabilidad en *P. carporrhizans*, especie de reproducción sexual obligada y heterotalina. La diversidad fue comparativamente mayor que la encontrada en líquenes con otros tipos de reproducción, como son la reproducción sexual obligada

homotalina y la reproducción asexual. La alta variabilidad hallada en *P. carporrhizans* se correspondió con una baja estructuración poblacional, nuestros resultados muestran tres poblaciones genéticas poco diferenciadas y sin estructura geográfica. El flujo génico entre las localidades macaronésicas y las localidades mediterráneas ha sido medido mostrando una alta conectividad. Este gran flujo génico y la distribución de los idiotipos del locus MAT unido a la gran diversidad genética y la baja estructuración poblacional demuestran la práctica panmixia de la especie. Esta panmixia no es total ya que los análisis espaciales realizados muestran aislamiento por distancia (r = 0.472) indicando una clina continua de diferenciación y los alelos geográficamente restringidos se dispersan menos que lo esperado bajo la asunción de panmixia. Las poblaciones macaronésicas resultaron menos variables y el flujo génico mostró migración unidireccional del Mediterráneo a las Islas Canarias. Los resultados son concordantes con un evento fundador seguido de pérdida de variabilidad por deriva génica y un patrón ecológico núcleo-periferia, donde los individuos del núcleo de distribución (más diverso) migran hacia la periferia a poblaciones receptoras que son dependientes de migración (menos diversas). Estas poblaciones periféricas tienen menor variabilidad genética que les confiere menor potencial adaptativo, y mayor riesgo de extinción local.

Conclusiones Altos niveles de diversidad permanecen ocultos en líquenes, enmascarados como especies crípticas, variabilidad poblacional o variabilidad intratalina. - Hemos demostrado la polifilia de *Punctelia rudecta* describiendo una nueva especie, *Punctelia guanchica* endémica de Canarias. – Hemos constatado la ocurrencia múltiple de fotobiontes en el seno del talo liquénico que se puede generar internamente o puede ser adquirida del exterior dependiendo de la especie. – Hemos constatado patrones de eyección y germinación de esporas diferentes entre *P. carporrhizans* y *P. quercina* así como diferencias en el desarrollo ontogenético que pueden deberse a la adaptación a diferentes hábitat. – Los marcadores microsatélites diseñados son aptos para el estudio de poblaciones genéticas y de ecología molecular. – Hemos confirmado la gran variabilidad y la baja estructuración genética de *P. carporrhizans* como se esperaba para una especie de reproducción sexual obligada y heterotalina. Nuestros resultados son consistentes con un evento fundador colonizando las Islas Canarias seguido de deriva génica que les hizo perder variabilidad.

SUMMARY

Microevolutive aspects and diversity in lichen co-symbionts

Introduction Lichens are paradigm organisms to study symbiosis for the stability of the symbiotic relationship between symbionts. Moreover, lichens are one of the best models to study ecological, biogeographical and reproductive facets due to the variety of reproductive modes and mating systems. In order to study the microevolution in lichens, or evolution at specific and infraspecific level, is necessary a precise species boundaries and species delimitation due to frequent appearance of cryptic species - morphologically indistinguishable - in lichens and in fungi in general. There are several theoretic species concepts but the General Lineage Concept obtains greater consensus between researchers. The integrative taxonomy joins multiple lines of evidence to satisfy the variety of species concept and species delimitation criteria. The Coalescent based methodologies has been very help to uncover hidden diversity in morpho species and even in polyphyletic species. Further, the use of appropriate molecular markers has been useful to uncover intra-thalline photobiont diversity, which has an adaptative advantage to changing environments. The diversity of the photobiont within lichen species and within a lichen thallus is highly influenced by the reproductive mode of the lichen species. To improve the knowledge about lichen symbionts and reproductive strategies, aposimbiotic cultures are recommended. Moreover, lichen axenic cultures have several applications including obtaining dyes and drugs used in textile and pharmaceutical industries, or sequence whole genome for further basic and applied research. For example, short sequences repeats (microsatellites) are highly variable genomic regions, and molecular markers designed to amplify these regions are used for population genetics and molecular ecology studies. In order to ensure which lichen symbiont is being analyzed, it is desirable obtain microsatellite markers from pure aposimbiotic culture.

Aims The general objective of this thesis is to investigate the diversity present in natural populations of lichen species and elucidate the implications of the genetic diversity in the speciation process in lichen co-symbionts.

Specifically, the thesis aims:

- Assessment of actual biodiversity within the *Punctelia rudecta* species complex and ascertain if it is a poliphyletic taxa, and elucidate the cryptic speciation.

- Evaluation of genetic diversity of *Trebouxia decolorans* photobiont within the lichen thallus.

- Isolate and culture in aposimbiotic conditions the mycobiont of *Parmelina*, to understand better some traits of their reproductive biology and ontogenetic development.

- Develop high resolution microsatellite markers specific to *P. carporrhizans* mycobiont for populational studies.

- Evaluate the genetic diversity and populational structure of *Parmelina carporrhizans* and their spatial distribution. Compare our results with previous studies in lichens with other reproductive modes.

Synthesis The four out of the five chapters included in this thesis dissertation are published in scientific journals indexed in the *Journal Citation Reports*, the chapter 3 was submitted to *The Lichenologist* and it is being reviewed. Of these, three are in the first quartile of their subject category (JCR).

We studied the biological diversity in *Punctelia rudecta* sensu lato, which biogeographical distribution pattern is at least partially coincident with Rand-flora pattern. However, *P. rudecta* is also present in the New World. For this research we selected three molecular markers of nuclear and mitochondrial DNA sequence regions (ITS, RPB1 and mtSSU) which are variable enough to study the variability at species level. They fulfill several characteristics such as one belong to nuclear region, while the other mitochondrial; and further one is spacer region while other one is transcribed, the other transcribed and traduced. To know the actual biological diversity within *P. rudecta* sensu lato we analyzing the information from molecular markers with statistical frameworks of species delimitation, some of them based on the coalescence. Moreover, to improve the species delimitations we used as many information sources as possible, implementing in an integrative taxonomic approach e.g. morphology, chemistry, ecology and geography. As result, we demonstrate the polyphyly of *P. rudecta.* We recognize at least four species within this nominal taxa describing a new endemic species from Canary Islands, *P. rudecta* sensu lato which was also typified, the other three species were epitypified in order to facilitate further works. Additionally we generated a new phylogeny that far from being complete was the best phylogeny published of the *Punctelia* genus until now comprising a third of the species. We

detected five major clades with statistical support which showed a chemical pattern based on medullary compounds.

We explored the within thallus variability of *Trebouxia decolorans* photobiont in *Anaptychia ciliaris* and *Xanthoria parietina* species. Employing high resolution microsatellite markers (SSR) we genotyped the photobiont from different portions within thallus checking if the identity of the photobiont was the same in any portion of the same thallus. Our results showed multiple photobionts in 60% of *Anaptychia ciliaris* thalli and 90% of *Xanthoria parietina* thalli. Moreover in *Anaptychia ciliaris* multiple alleles were found in the multilocus genotypes of several thallus portions. Meanwhile in *X. parietina* photobionts had higher rarefacted allelic richness and less related alleles. Acquisition of different photobiont lineages was discussed as due to *de novo* generation of the diversity by somatic mutation. While in the case of *X. parietina* the higher allelic richness and unrelated alleles suggests also the environmental uptake of new photobiont either at the start of the symbiotic association or during the lifetime of the thallus. Our results altogether with previous studies suggest that the frequency of co-occurrence of multiple photobiont is much higher than was previously suspected in lichens with Trebouxioid photobionts.

We developed aposymbiotic cultures of the mycobionts of *Parmelina carporrhizans* and *Parmelina quercina*. Aposymbiotic cultures of lichen mycobiont have the interest of several aplications in textile and pharmacological industries and also in basic science and academic knowledge. We isolated the mycobiont of *Parmelina carporrhizans* and *Parmelina quercina* applying the inverted plate dish technique and cultured in aposymibiotic conditions with two aims consisting of the comparison between both species; (i) the ontogenetic development from spores to mature mycelia, and (ii) different aspects of the reproductive biology of both species. In our work we measured several parameters as spores production by mature apothecia, number of ejected spores and percentage of spore germination taking into account the apothecia size class. We obtained high number of spores ejected by apothecia, hundreds in *P. carporrhizans* and thousands in *P. quercina* and higher number of spores ejected in large apothecia than in smaller in both species. The mixed model (species-thalli) was the best to explain the observed differences, the specific differences were significant but the ejection pattern was also influenced by individual effect. Germination was the most different parameter between species, while most of the *P. carporrhizans* spores germinated (90%), just a few of *P. quercina* did it (0.5%). Moreover most of the germinated spores of *P. quercina* reached abortive stages a few days after germination

and mycelia formation was unusual. No significant differences were observed in germination rates between large and small apothecia. Growth rates in *P. carporrhizans* were evaluated in three different culture media and discussed in comparison with other lichen mycobionts. Both species produced phenolic compounds which provided dark pigmentation to mature mycelia.

After successfully culturing the *P. carporrhizans* mycobiont and obtaining enough biomass, we sequenced the genome of the species using Next Generation sequencing, Illumina platform. We designed microsatellite markers with msatcommander software selecting microsatellite loci containing from 15 to 20 repetitions. These genetic markers were tested across samples of Parmelina species to chose the most effective to amplify the samples and more variability recover within *Parmelina carporrhizans* populations.

The population study of *Parmelina carporrhizans* analyzed by microsatellite markers and MAT genes showed a high variability measured as allelic richness and absence of clonality. This is most probably due to the reproductive mode, which is obligately sexual and heterothalline. The variability found in *P. carporrhizans* was higher compared with lichens with other reproductive modes as homothalline obligate sexual or asexually reproducing lichens. We found three genetic populations with low differentiation that do not show a geographic structure. We measured high gene flow between Mediterranean and Macaronesian populations, which together with the balanced MAT locus idiomorph distribution between and within populations suggest almost panmixia in the species. Our results are according to the ecological edge-core pattern, where individuals from the more genetic diverse core populations migrate to the periphery to receptor populations which are migration dependent. The peripheral Macaronesic populations showed lower diversity measured as allelic richness and diminished adaptative potential being sensitive to local extinction.

Conclusions High levels of diversity remain hidden in lichen co-symbionts, masked as cryptic species, population variability or intrathalline variability. The geographical isolation showed relevance for population differentiation and speciation. - We demonstrated the polyphyly of *Punctelia rudecta* finding several species within this nominal taxa and describing the new endemic species *Punctelia guanchica.* - We showed that photobiont is more variable within thallus than expected, with multiple photobiont lineages generated by somatic mutation and additionally environmental uptake can add more variability or specificity – We addressed the patterns of spore ejection and germination, ontogenic development and growth capacity between

Parmelina carporrhizans and *P. quercina*, reporting different reproductive behavior that suggest different reproductive ecological strategies. – The whole genome sequence of *P. carporrhizans* has been used to develop the microsatellite markers, which are appropriate for population level and ecological studies. – We confirmed that *P. carporrhizans* has high genetic variability and low populational structure as expected for an obligated sexually reproducing lichen. Our results are consistent with a founder event colonizing Canary Islands followed by diversity losing by genetic drift.

INTRODUCCIÓN GENERAL

La diversidad biológica es la variedad de formas de vida o número de especies y la diversidad genética es la variabilidad en la secuencia de la molécula de ácido desoxirribonucleico (ADN) entre especies y en el seno de cada una de las especies. La evolución es el mecanismo de cambio en el tiempo que genera y transforma la diversidad genética en diversidad biológica. En un sentido pragmático se divide en macroevolución cuando se estudia periodos de tiempo grande y categorías supraespecíficas y microevolución cuando se estudia en periodos de tiempo corto y en rango de especie o inferior. La microevolución es también la parte más visible de la evolución; como ejemplo microevolutivo tenemos el caso clásico de selección del melanismo en la mariposa nocturna *Biston betularia* (Berry 1990).

Los líquenes son organismos compuestos por al menos dos simbiontes, que llamamos micobionte y fotobionte y componen el cuerpo del organismo que se conoce como talo. El micobionte es un hongo filamentoso generalmente de la división Ascomycota y el fotobionte suele ser un alga unicelular de la división Clorophyta o más ocasionalmente una cianobacteria; raramente se da la coexistencia de fotobiontes de ambos tipos. En todo caso la simbiosis liquénica podría ser más compleja, si se acepta como tales integrantes de la misma otros organismos que se han encontrado cohabitando en talos detalladamente investigados (levaduras, comunidades bacterianas etc.) (Grube *et al.* 2009; Grube & Berg 2009, Sprilbille *et al*. 2016). Aunque los líquenes son una asociación estable la relación entre los cosimbiontes no es igualitaria (Lücking *et al.* 2009). La reproducción del talo como un todo puede ser de tipo sexual o asexual pero solo el micobionte es capaz de reproducirse sexualmente, llegando incluso a inhibir la reproducción del fotobionte (Honegger 1996). Por este motivo el código de nomenclatura establece que el nombre (y la sistemática o taxonomía) de la especie de un líquen se refiere al micobionte como establece el Código de Nomenclatura vigente (McNeil *et al.* 2012).

La especie es la unidad del sistema taxonómico y tanto en aspectos conceptuales como en su delimitación ha estado sujeto a discusión, especialmente desde que Darwin publicara *On the Origin of Species by Means of Natural Selection* (1859). Actualmente hay un consenso amplio en considerar que una especie es un linaje que evoluciona de forma independiente (Coyne & Orr 2004). Sin embargo, hay al menos 29 conceptos de especie (SC) diferentes, que se diferencian por el criterio que utilizan para establecer la independencia evolutiva (De Queiroz 2005). La solución adoptada fue integrar los distintos conceptos de especie existentes, y así surge el Concepto General de Linaje (GLC) (De Queiroz 1998). Inicialmente el concepto de especie era morfológico (MSC) distinguiendo las especies por apariencia. Desde mediados del siglo pasado prevaleció el concepto biológico de especie (BSC) cuyo criterio es el aislamiento reproductivo. En botánica es relevante el criterio ecológico de especie (MSC) que pone énfasis en el nicho ecológico. Y uno de los más populares en la actualidad es el concepto filogenético de especie (PSC) cuyo criterio se basa en las diferencias de secuencia en regiones concretas de la molécula de ADN. A pesar de la prevalencia de los datos moleculares, las limitaciones intrínsecas que tienen estos datos y su análisis, hacen imposible la superación total de los anteriores conceptos de especie por el PSC (Leavitt *et al.* 2016). En la práctica establecer correctamente los límites de las especies es imprescindible para una buena clasificación de las especies y para entender la evolución de las mismas (Leavitt *et al.* 2016).

A la formación de especies nuevas la llamamos especiación, y es el proceso más importante de la microevolución. En los términos del análisis filogenético, se interpreta que las especies se originan principalmente por el mecanismo de cladogenesis, en el que una especie ancestral se divide en dos especies hermanas. En el proceso de especiación por cladogenesis podemos apreciar tres etapas (véase la figura más abajo). 1) Etapa de mosaico genético: se genera el polimorfismo. 2) Etapa de diferenciación: poblaciones genéticas desarrollan aislamiento reproductivo parcial (aquí los linajes pueden ser reconocidos como especie según algunos de los “species criteria” (SC). 3) Etapa de independencia: cuando los linajes independientes conforman efectivamente nuevas especies.

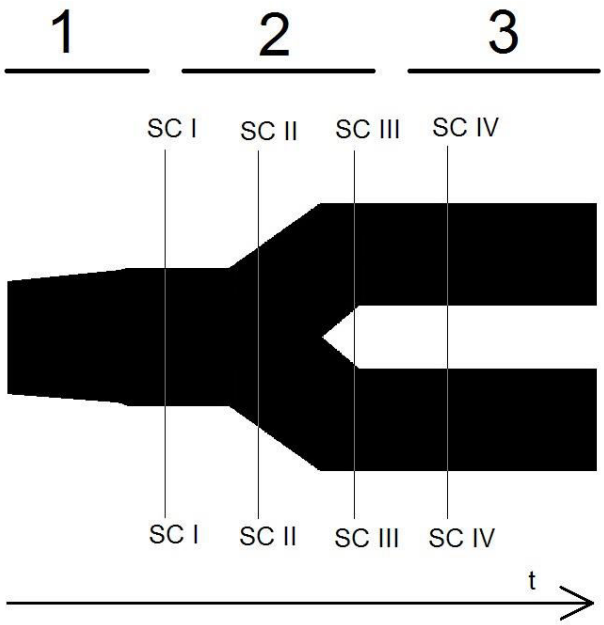

Figura 1 Esquema de especiación por anagénesis.

1) En la base hay una especie única que va generando diversidad

2) después se bifurca en el proceso de especiación

3) generando dos especies al final del proceso (3).

Los caracteres distintivos que separan ambas especies según concepto de especie (SC "species criteria") se van adquiriendo gradualmente.

Una vez que los linajes alcanzan el rango de especies, las características secundarias relativas a los conceptos de especie pueden o no estar presentes. Por ejemplo en los linajes parafiléticos; Así en *Parmelina tiliácea* con *P. pastillifera,* (Núñez-Zapata *et al.* 2015) al existir un rasgo morfológico de valor o carácter diagnóstico son buenas especies según el MSC, pero no son buenas especies según el PSC, porque los caracteres moleculares tienen dificultades para distinguir ambas especies con algunos de los marcadores de uso habitual. El ejemplo opuesto se da con gran frecuencia en hongos y es lo que conocemos como especies crípticas, que por la ausencia de caracteres morfológicos, no son posibles de distinguir por el MSC (Crespo & Lumbsch 2010), como en el caso de *P. tiliácea* y *P. cryptotiliacea (*Núñez-Zapata *el al.* 2011) pero si son distinguibles según el PSC.

Los distintos conceptos de especie, las especies parafiléticas y las especies crípticas suponen una dificultad para el reconocimiento taxonómico y para identificar las especies, por ello se han estado desarrollando metodologías empíricas para delimitar unas especies de otras en casos de especial dificultad. Así hay una serie de metodologías que se basan en el análisis informático de la información molecular (Leavitt *et al.* 2015a). Con objeto de alcanzar la máxima resolución al establecer los límites de las especies nace la taxonomía integrativa (German National Academy of Sciences Leopoldina 2014). Esta metodología de síntesis pretende la combinación de las distintas metodologías de delimitación de las especies y otros taxones, con datos morfológicos, químicos, ecológicos y tantos otros criterios de evidencia como sea

posible. Particularmente en los hongos, el reconocimiento de especies crípticas ha ayudado a tomar conciencia de la biodiversidad real hasta tal punto que se ha llegado a reconocer casos en los que 126 especies que eran consideradas como una única (Lücking *et al.* 2014). Antes del reconocimiento de especies crípticas en líquenes se pensaba que la mayoría de las especies eran de distribución cosmopolita, sin embargo ahora se sabe que una gran parte de ellas eran en realidad un conjunto de especies de distribución local (Crespo & Pérez-Ortega 2009, Lumbsch & Leavitt 2011).

Las metodologías de delimitación de especies también se han aplicado al estudio de fotobiontes de líquenes, donde se han encontrado gran diversidad oculta (Skaloud y Peksa 2010; Sadowska-Des *et al.* 2014; Leavitt *et al.* 2015a). Sin embargo, en la delimitación de especies en fotobiontes se hace difícil llegar a conclusiones taxonómicas debido a la falta de suficiente conocimiento derivada de la falta de datos sistemáticos. Ej. Leavitt *et al.* (2015) reconoció 48 nuevas OTU que no pudieron ser formalizadas como nuevas especies. La aún menor abundancia de caracteres morfológicos en los fotobiontes y/o la dificultad de su estudio (pues a veces se necesita de un microscopio electrónico para localizar caracteres) provocó la subestimación de la diversidad cuando el análisis se basaba en datos morfológicos (Skaloud & Peksa 2010). Los estudios moleculares han permitido descubrir parte de la diversidad que estaba oculta. De hecho, mediante las herramientas moleculares se ha podido poner de manifiesto, no solo diversidad taxonómica de los fotobiontes liquenicos en general sino también cierta diversidad en un líquen concreto, como variabilidad intratalina. En *Ramalina farinácea* se descubrió, gracias a secuencias de la subunidad grande ribosómica (LSU) del cloroplasto, que había más de un fotobionte coexistiendo en el mismo talo (las especies putativas TR1 y TR9); curiosamente esto pasaba inadvertido al analizar la secuencia del espaciador transcrito interno (ITS) (del Campo *et al.* 2010) que es el marcador molecular más frecuentemente utilizado a nivel de especie e inferior (Schoch *et al.* 2012). Ambos fotobiontes encontrados coexistiendo en un talo fueron cultivados y se estudiaron en ellos las diferencias de comportamiento fisiológico (del Hoyo *et al.* 2011), argumentándose que la coexistencia en el mismo talo aportaba ventajas adaptativas al complementarse ecofisiológicamente (Casano *et al.* 2011). Conocer la diversidad real del fotobionte nos permite profundizar en como de selectiva y específica es la relación entre simbiontes, y es un dato importante para poder inferir el potencial adaptativo, ya que este está asociado al grado de diversidad genética (Pauls *et al.* 2013, Stiebens *et al.* 2013, Singh *et al.* 2016). El progreso en las técnicas moleculares mediante el desarrollo de marcadores genéticos altamente polimórficos de gran resolución, como los

microsatélites, repeticiones de secuencias simples (SSR), permiten conocer la variabilidad no solo a nivel de especies putativas sino a nivel poblacional y de diferencias individuales. Los fotobiontes de líquenes han sido estudiados con microsatélites en solo dos especies, ambas con reproducción asexual, y se encontró congruencia genética entre ambos simbiontes. En *Parmotrema tinctorum* se encontró más de un fotobionte probablemente de distinta especie en casi todos los talos estudiados (Mansournia *et al.* 2012) y en el 1.6 % de los talos de uno de los estudios realizados en *Lobaria pulmonaria* (Werth *et al.* 2006, Dal Grande *et al.* 2014).

Cabe esperar una mayor diversidad en los fotobiontes de especies de líquenes que tienen reproducción sexual que en los que se reproducen de forma asexual. Porque la reproducción por medio de esporas sexuales requiere la reliquenización y captación de nuevo fotobionte cada generación. Al contrario, en los líquenes asexuales la transmisión del fotobionte es vertical. La ocurrencia de fotobiontes Trebouxioides de vida libre está muy discutida y se ha sugerido que la captación por parte del micobionte se produce a partir de talos maduros o propágulos vegetativos de dispersión de la misma especie o del mismo grupo funcional (Beck *et al.* 1998). Los grupos funcionales (*ecological guild*) en líquenes consisten en especies de líquenes compartiendo fotobionte y hábitat, y se supone que este hecho confiere ventaja adaptativa, esto se ha visto especialmente en especies saxícolas (Peksa & Skaloud 2011). Otra posibilidad es que la captación del fotobionte sea a partir de comunidades de algas que colonizan la superficie de los líquenes (Muggia *et al.* 2013).

El estudio de las estrategias reproductoras en líquenes es complejo especialmente por la falta de trazabilidad de las esporas y propágulos de dispersión en la naturaleza y la imposibilidad de cerrar el ciclo biológico en cultivo. Los cultivos microbiológicos que se realizan para la investigación de líquenes se hacen aislando y cultivando solo uno de los dos simbiontes (cultivo aposimbiótico). Se han realizado trabajos de resíntesis cultivando por separado ambos simbiontes y al unirlos posteriormente se han recreado estadios tempranos de liquenización (Brunauer *et al.* 2007) pero sin llegar a obtener un talo maduro ni estructuras reproductivas. Los cultivos aposimbióticos se han visto como una buena herramienta para aportar conocimiento acerca del ciclo biológico y las estrategias reproductivas de líquenes. Sirvan como ejemplo estudios de selectividad entre simbiontes y caracterización de las moléculas de reconocimiento (Bubrick & Galun 1980, Schaper & Ott 2003), y estudios de caracterización de genes que determinan el sexo en hongos, locus MAT (Scherrer *et al.* 2005; Singh *et al.* 2012). Los cultivos aposimbióticos son también una buena

herramienta para conocer el desarrollo ontogenético y descubrimiento de metabolitos secundarios (Fazio *et al.* 2009) y sus aplicaciones farmacológicas y médicas (Molina *et al.* 2015; Fernández-Moriano *et al.* 2015).

Una de estas aplicaciones de los cultivos aposimbióticos es la secuenciación de genomas completos que a su vez posibilitan estudios filogenéticos, búsqueda de genes concretos y desarrollo de nuevos marcadores genéticos para estudios poblacionales. Todavía está en una etapa inicial la secuenciación de genomas de líquenes y la mayoría de los genomas secuenciados aún no han sido publicados en bases de datos especializadas (en la presente tesis hemos secuenciado el genoma de *Parmelina carporrhizans* y no ha sido publicado). Sin embargo, sí que han proliferado los trabajos publicando nuevos marcadores altamente polimórficos para ser aplicados a estudios poblacionales, sobretodo microsatélites. Los microsatélites son secuencias que consisten en la repetición de secuencias de pocos nucleótidos que proporcionan una gran tasa de polimorfismo en los segmentos genómicos; característicamente este polimorfismo se genera de acuerdo al neutralismo de Kimura. Muchos de los trabajos de desarrollo de microsatélites para trabajar con líquenes lo hacen a partir de cultivo aposimbiótico (Widmer *et al.* 2010, Dal Grande *et al.* 2010; Dal Grande *et al.* 2013; Werth *et al.* 2013; Guzow-Krzemińska *et al.* 2013, Lindgren *et al.* 2016) para evitar desarrollar marcadores para el simbionte equivocado. De hecho tal riesgo se ha consumado en algunas investigaciones: Por ejemplo Walser (2003) diseñó marcadores para micobionte a partir de talo (Walser 2003) y posteriormente se pudo constatar que algunos eran específicos del fotobionte (Widmer *et al.* 2010). El desarrollo de marcadores genéticos fiables y de gran variabilidad son condiciones necesarias para la más alta fiabilidad de los estudios de genética de poblaciones con organismos tan complejos como los líquenes.

La genética de poblaciones es una disciplina que juega un rol central en la comprensión de lo que entendemos como microevolución. Estudios microevolutivos, como la mutación, la selección natural, la deriva genética y la migración en poblaciones mendelianas se abordan desde esta perspectiva. Es por tanto una herramienta apropiada para estudiar los procesos evolutivos en las etapas tempranas de especiación previas a la formación de especies nuevas.

OBJETIVOS

-Mejorar el conocimiento que tenemos de los simbiontes que forman el talo liquénico, su diversidad y su desarrollo ontogenético.

- Estudiar la diversidad presente en las poblaciones de las especies de líquenes y sus implicaciones en la especiación. Haciendo hincapié en las metodologías que nos permiten distinguir taxones infraespecíficos de especies crípticas.

Los objetivos específicos de esta tesis son:

- Evaluación de la diversidad biológica real en un complejo de especies tomando como modelo *Punctelia rudecta*, demostrar si es un taxón polifilético y elucidar la especiación criptica.

- Evaluación de la diversidad genética del fotobionte *Trebouxia decolorans* en el talo liquénico.

- Cultivar en condiciones aposimbióticas el micobionte de *Parmelina* para conocer mejor algunos aspectos de su desarrollo ontogenético y su reproducción.

- Desarrollar marcadores genéticos de microsatélites de alta resolución específicos del micobionte de *P. carporrhizans* para realizar estudios poblacionales.

- Estudiar la diversidad genética y la estructura poblacional de *Parmelina carporrhizans* en relación a su modo reproductivo.

CAPITULO I / CHAPTER I

An Integrative Approach for Understanding Diversity in the *Punctelia rudecta* Species Complex (Parmeliaceae, Ascomycota)

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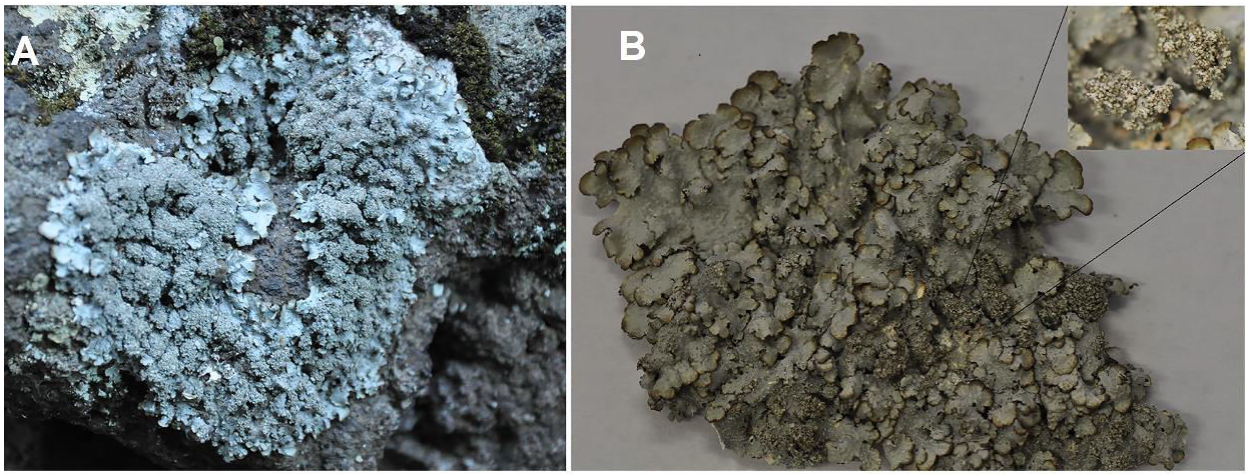

Punctelia guanchica (A) showing habitat of *Punctelia guanchica*, (B) habit of *P. guanchica* with enhanced detail of isidia in the right upper corner of the picture.

Abstract

High levels of cryptic diversity have been documented in lichenized fungi, especially in *Parmeliaceae*, and integrating various lines of evidence, including coalescent-based species delimitation approaches, help establish more robust species circumscriptions. In this study, we used an integrative taxonomic approach to delimit species in the lichen-forming fungal genus *Punctelia* (*Parmeliaceae*), with a particular focus on the cosmopolitan species *P. rudecta*. Nuclear, mitochondrial ribosomal DNA and protein-coding DNA sequences were analyzed in phylogenetic and coalescence-based frameworks. Additionally, morphological, ecological and geographical features of the sampled specimens were evaluated. Five major strongly supported monophyletic clades were recognized in the genus *Punctelia*, and each clade could be characterized by distinct patterns in medullary chemistry. *Punctelia rudecta* as currently circumscribed was shown to be polyphyletic. A variety of empirical species delimitation methods provide evidence for a minimum of four geographically isolated species within the nominal taxon *Punctelia rudecta*, including a newly described saxicolous species, *P. guanchica,* and three corticolous species. In order to facilitate reliable simple identification for biodiversity, conservation, and air quality bio-monitoring research, these three species have been epitypified, in addition to the description of a new species.

Keywords: *Punctelia rudecta*, species complex, species delimitation, taxonomy

Introduction

Circumscribing species in groups with relatively simple and variable morphologies is difficult. Lichen-forming fungi are a prime example of an organismal group where the use of molecular sequence data has challenged many traditional, phenotype-based species circumscriptions. A growing body of evidence indicates that traditionally circumscribed species of lichen-forming fungi commonly mask a substantial amount of species-level diversity (Divakar *et al.* 2013). So-called cryptic species with no obvious morphological differences are common in these organisms, especially in *Parmeliaceae*, one of the largest and most morphologically diverse families of lichenized fungi (Crespo *et al.* 2010; Thell *et al.* 2012; Divakar *et al.* 2013; Kraichak *et al.* 2015). At least 80 unrecognized species-level lineages have been identified in this family so far (Crespo & Lumbsch 2010). Although some species of lichen-forming fungi have been shown to be truly widespread (Lindblom & Sochting 2008: Lumbsch *et al.* 2008, Fernandez-Mendoza *et al.* 2011; Perez-Ortega *et al.* 2012, Wirtz *et al.* 2012; Del Prado *et al.* 2013; Leavitt *et al.* 2013; Fernandez-Mendoza & Printzen 2013), in other cases striking biogeographical patterns have been revealed by revised species delimitation in nominal species that were previously assumed to be cosmopolitan or widely distributed (Argüello *et al.* 2007; Elix *et al.* 2009; Otálora *et al.* 2010; Sérusiaux *et al.* 2011; Amo de Paz *et al.* 2012; Parnmen *et al.* 2012; Fernandez-Mendoza & Printzen 2013; Moncada *et al.* 2014).

An ongoing appeal to researchers to assess species boundaries using multiple lines of evidence (phylogenetics, population genetics, comparative morphology, development, ecology, etc.) has resulted in an increased emphasis to utilize an integrative framework in species delimitation studies (Fujita *et al.* 2012). While in practice integrative approaches for species delimitation fall across a broad spectrum, ranging from verbal and qualitative assessments of data classes to quantitative methods that allow different data types to contribute to statistical species delimitation, any study linking different kinds of data to support hypotheses of species boundaries, including placing morphological characters onto a molecular phylogeny, can be considered integrative (Leavitt *et al.* 2015). Although an iterative process involving the comparison of a molecular phylogeny and morphological data provides a simple approach for

species delimitation, integrative taxonomy should combine as many independent lines of evidence as available to reach higher resolution without lack of reliability (Miralles & Vences 2013).

Punctelia is a medium-sized genus in *Parmeliaceae* described by Krog (Krog 1982), with approximately 45 currently accepted species (Thell *et al.* 2012). Species in this genus are characterized by the presence of unciform to filiform conidia, punctiform pseudocyphellae and simple rhizines. The genus has a temperate to subtropical distribution with centers of distribution in the Neotropics and Africa. In a previous study in the genus *Punctelia* (Crespo *et al.* 2004), we found evidence that *P. rudecta* is not monophyletic, and in this study we focus on delimiting species boundaries in this widespread taxon. *P. rudecta* has a subcosmopolitan distribution, being known from North and South America, Africa, and Asia (Krog & Swinscow 1977; Krog 1982; Hawksworth *et al.* 2011). In spite of its broad, intercontinental distribution, it appears to be absent from Australasia (Elix & Johnston 1988) and records from Europe are dubious (Hawksworth *et al.* 2008). Here we assembled a molecular dataset from *P. rudecta* specimens collected from different geographical regions to address its circumscription and test for the potential of geographically isolated, distinct lineages.

The aims of this study are to demonstrate that *Punctelia rudecta* as it is currently circumscribed is poliphyletic and elucidate how many species are masked within this nominal taxon. We collected specimens throughout the known distribution of the species for molecular and morphological evaluation. We focus our efforts on integrating multiple lines of evidence, including molecular phylogenies, coalescent-based species delimitation methods, morphology, ecology, chemistry and biogeography, to assess species circumscription. In addition to evaluating species boundaries within *P. rudecta* s. lat., we also provide the most comprehensive phylogeny to date for *Punctelia* genus, including one-third of the known species.

Materials and Methods

Taxon sampling For this study, we sampled a total of 87 *Punctelia* specimens, representing 16 traditionally circumscribed species (Supplementary Table 1). Punctelia specimens were collected from all continents, with the exception of Antarctica where the species is not known to occur. Overall, our sampling focused on the *P. rudecta* s. lat (n = 40)

and included specimens from Canada (1), Canary Islands (10), Chile (2), China (1), India (2), Japan (4), Kenya (5), and USA (15) (Supplementary Table 1). Sequence data was generated from 48 *Punctelia* specimens with vouchers in MAF-Lich and combined with sequences from 39 *Punctelia* specimens downloaded from GenBank (Supplementary Table 1). In addition to *Punctelia* samples, *Flavopunctelia flaventior* and *F. soredica* were used as outgroups, based on results from previous phylogenetic studies (Blanco *et al.* 2006; Lumbsch *et al.* 2008; Crespo *et al.* 2010).

All *Punctelia* specimens were examined under a stereomicroscope (Nikon SMZ1000) to study morphological characters (Supplementary Table 2). Anatomical characters were studied with a Zeiss Axioscope using hand-cut sections of ascomata and conidiomata. Secondary metabolites were identified by thin-layer chromatography (TLC) following standardized procedures using solvent systems B and C (Lumbsch 2002).

DNA extraction and amplification Small thallus fragments were excised under a dissecting microscope and crushed with sterile glass pestles in liquid nitrogen. Total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with slight modifications as previously described (Crespo *et al.* 2001). We generated sequence data from three loci: the nuclear ribosomal internal transcribed spacer region (nuITS), the mitochondrial small subunit (mtSSU), and the nuclear protein coding locus RNA polymerase II largest subunit (RPB1). PCR amplifications were performed using the following primers: 1) ITS1-F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) for the nuITS rDNA region; 2) mrSSU1 and mrSSU3 (Zoller *et al.* 1999) for the mtSSU; and 3) gRPB1-C (Matheny *et al.* 2002) and RPB1-MH-F (Leavitt *et al.* 2012) for the RPB1. PCR amplifications were conducted in 25 µL with 0.5 Ml of Taq polymerase. In some cases where standard PCR failed to amplify target loci, we used Ready-To-Go PCR Beads (GE Healthcare) following manufacturer's recommendations. PCR amplifications were carried out following conditions: one initial heating step of 4 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C (ITS), 54°C (mtSSU) and 90 s at 72°C, and a final extension of 10 min at 72°C. The PCR amplification for RPB1 was carried out following the conditions: one initial heating step of 10 min at 94°C,

followed by 38 cycles of 45 s at 94°C, 50 s at 56°C and 1 min s at 72°C, and a final extension of 5 min at 72°C.

PCR products were visualized on 1% agarose gel and stained with SYBR safe and cleaned using ExoSAP-IT (USB, Cleveland, Ohio, USA), following manufacturer's instructions. Complementary strands were sequenced from cleaned PCR products with the same primers used for amplifications. Sequencing reactions were performed using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on a 3730 DNA analyzer (Applied Biosystems) at the Unidad de Genómica (Parque Científico de Madrid).

Alignments and phylogenetic analyses Sequences of each locus were aligned using MAFFT 7 (Katoh *et al.* 2009), with the 'auto' mode threshold and default settings. Subsequently ambiguously aligned positions representing were removed using the Gblocks (Castresana 2000) web server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html), implementing the options for a "less stringent" selection of ambiguous regions. Althought Gblocks was observed to worsen the accuracy of phylogenetic tree reconstruction has Little inpact under light filtering (up to 20% of aligned positions) (Tan *et al.* 2015) we remove 1.3% of aligned positions. Exploratory analyses revealed no significance difference on phylogenetic tree reconstruction. Individual gene alignments were concatenated using Mesquite 2.5 (Maddison & Maddison 2011). Exploratory analyses of single-locus phylogenies did not show any well-supported conflicts (bootstrap > 70% (Amo de Paz *et al.* 2012)) among topologies, and the concatenated dataset was used to infer phylogenetic relationships. Nucleotide substitution models for each locus were chosen using jModeltest (Darriba *et al.* 2012): SYM for the ITS marker, HKY85 for mtSSU and K80 for RPB1. These models were specified as imput options in both the Bayesian gene tree inference and the species tree reconstructions. Maximum likelihood (ML) analyses were performed using RAxML 8.1.11 (Stamatakis 2006) and Bayesian tree samplings were done with MrBayes 3.2 (Ronquist *et al.* 2012). The ML analysis was performed using the GTRGAMMA model, partitioning the combined 3-marker dataset by locus, and nodal support was assessed using non-parametric bootstrapping carried out for 1000 pseudoreplicates (Stamatakis *et al.* 2008). For the Bayesian tree sampling, the concatenated three-locus data set was partitioned as described in the ML analysis, specifying the best fitting model, and allowing

unlinked parameter estimation and independent rate variation. Two parallel runs comprised of 10,000,000 generations, starting with a random tree and employing eight simultaneous chains, were executed. Posterior probabilities (PP) were estimated by sampling trees using a variant of Markov Chain Monte Carlo (MCMC) method. Every 1000th tree was sampled to avoid sample autocorrelation. Based on the likelihood profile, the first 25% trees were discarded as burn in. A 50% majority-rule consensus tree with average branch lengths was computed from the remaining trees, using the sumt option of MrBAYES. Only clades with bootstrap support equal or above 70% under ML and PP equal to or above 0.95 in a Bayesian framework were considered as supported. Phylogenetic trees were visualized using the program FigTree 1.4.0 (Rambaut 2009).

Alternative hypothesis testing Since *Punctelia rudecta* was recovered as polyphyletic in our analyses (see Results), we tested whether our data is sufficient to reject monophyly of this species as currently circumscribed. For the alternative hypothesis testing, we compared constrained topologies with unconstrained topologies obtained in RaxML (under GTRGAMMA substitution model) using two different methods: i) Shimodaira-Hasegawa (SH) test (Shimodaira & Hasegawa 2001) and ii) expected likelihood weight (ELW) test (Strimmer & Rambaut 2002). The SH and ELW test were performed using Tree-PUZZLE 5.2 (Schmidt *et al.* 2002) with the concatenated data set, comparing the best tree agreeing with the null hypotheses, and the unconstrained ML tree. These trees were evaluated in Tree-PUZZLE using the GTR+I+G nucleotide substitution model. We investigated two constrains to test the polyphyly of *P. rudecta* species, 'constraint 1' consisted of (*P. rudecta* + *P. guanchica* + *P. ruderata* + *P. aff. rudecta*). 'Constraint 2' consisted of (*P. rudecta* + *P. guanchica* + *P. ruderata* + *P. aff. rudecta* + *P. toxodes* + *P. missouriensis*) corresponding to species belonging to clades 'A' and 'B' and excluding the subclade of the sorediate species *P. subrudecta* and *P. perreticulata*.

Species delimitation analyses It has been shown repeatedly that different methods for empirical species delimitation can yield incongruent results (discussed in Carstens *et al.* 2013), which is among others due to simplifying assumptions that each of the methods make and different potentials to detect cryptic lineages. Therefore, we explored a variety of methods

implemented for both single locus data and otherapproaches for multilocus sequence data, for the single locus approaches we used the ITS dataset matrix because ITS marker has been adopted as the primary barcode for fungi (Schoch *et al.* 2012). Ultimately, we used five different methods to delimit species in the Punctelia rudecta group. First, we used the genetic distance-based method "Automatic Barcode Gap Discovery" (ABGD) which takes single locus matrix as input (Puillandre *et al.* 2012). Two tree-based species delimitation methods– the generalized mixed Yule coalescent model (GMYC) which takes ultrametric single locus as input (Pons *et al. 20*06; Monaghan *et al.* 2009) and the Poisson tree process model which takes single locus as input (PTP) (Zhang *et al.* 2013) were also used for species delimitation. Finally, two coalescent-based species tree-based methods species delimitation using species tree using maximum likelihood (spedeSTEM) which takes single locus trees as input, merging all in one to analyze (Ence & Carstens 2011) and "Bayesian Phylogenetics and Phylogeography" (BP&P) which takes multilocus data and a prior species tree as input (Yang & Rannala 2010) were implemented to test circumscribe species.

For ABGD we used default parameters except for using a P_{max} at 0.01 and a relative gap width of 0.5. For the GMYC analyses, outgroup samples were excluded from the ITS data set with drop tip in ape (Paradis *et al.* 2004). A chronogram was calculated from theML tree using the penalized likelihood method (Sanderson 2002) as implemented in the chronopl command in ape (Paradis *et al.* 2004; RDT 2008). The GMYC method requires a fully dichotomous chronogram and thus we used multdivtime (Paradis *et al.* 2004) to convert our chronogram into a fully dichotomous chronogram with internal branches of length zero, where appropriate. This modified chronogram was then analyzed using the gmyc function in the SPLITS packagein R (version 2.10, www.cran.r-project.org), employing the single ($GMYC_{single}$) and multiple threshold ($GMYC_{multiple}$) methods. The PTP analysis was done on the web server of the Exelixis Lab (http://species.h-its.org) using default settings. For the spedeSTEM multilocus analysis we used the web server of the Carstens Lab (https://spedestem.osu.edu/runspedestem) with default settings. We used BPP 3.1 (Yang & Rannala 2010) which utilizes rjMCMC algorithm and incorporates nearest-neighbor interchange algorithm eliminating the need for a fixed species tree allowing changes in the species tree topologies. To run BPP 3.1 we used the Bayesian species tree performed in *Beast as a prior.

The data was analyzed with both algorithm0 and algorithm1 with gamma prior G~ (2, 1000) and the other parameters were assigned to the Dirichlet prior (Carstens & Dewey 2010) Each analysis was run twice to confirm the consistency between runs and the results showed in this work corresponds to the analysis performed with algorithm1.

Bayesian species tree In order to delimit species in a multispecies coalescent framework, we performed a species tree analysis using *Beast as implemented in Beast v.1.8.0 (Drummond & Rambaut 2007; Heled & Drummond 2010) and analized in BP&P where the nodal support (e.g., 'speciation probabilities') were calculated. The *Punctelia* species tree topology was estimated using a rate-calibrated multilocus coalescent-based species tree approach in *Beast. We used a starting tree based on putative species identified from spedeSTEM, where *P. hypoleucites*USA was treated as *P. rudecta* s.str. and *P. hypoleucites*_from Cuba treated as *P. caseana* (see supplementary data). As unique modification we treated *P. missouriensis* and *P. aff. rudecta* as single lineages to in order to test this potentially interesting lineages. Using an uncorrelated relaxed lognormal clock (Drummond *et al.* 2006) we selected a birth-death model for the species tree prior; the population size model was set to piecewise linear and constant root. Molecular evolution rates for the ITS was set at 2.43×10^{-9} substitution/site/ year (s/s/y) (Leavitt *et al.* 2012) and 1.76×10^{-9} s/s/y for RPB1 (Amo de Paz *et al.* 2011). Two independent MCMC analyses were run for a total of 100 million generations (sampling every 2000 steps and excluding the first 25 million generations of each run as burn-in). Convergence was assessed by ensuring that standard deviations of split frequencies between runs approached zero, visualizing split probabilities in "Are we there yet?", AWTY (Nylander *et al.* 2008) and comparing summarized tree topologies from separate runs. After removing the first 20% of the samples as burn-in, all runs were combined to generate posterior probabilities of nodes from the sampled trees using TreeAnnotator v1.8.0 (Rambaut & Drummond 2009). Mean node age and 95% highest posterior density (HPD) were mapped on the maximum clade credibility tree. The species tree produced by *BEAST was subsequently used as the guide tree for inferring speciation probabilities in BP&P (Yang & Rannala 2010).

Nomenclature The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS ONE article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

In addition, new names contained in this work have been submitted to IndexFungorum from where they will be made available to the Global Names Index. The unique IndexFungorum number can be resolved and the associated information viewed through any standard web browser by appending the IndexFungorum number contained in this publication to the prefix http://www.indexfungorum.org.

Results

Phylogenetic analyses and alternative hypothesis testing We assembled a total of 213 DNA sequences from 89 specimens, including 134 new sequences generated for this study (Supplementary Table 1). The 476 bp ITS dataset was comprised of 89 sequences, with a nucleotide diversity of 0.043; the mtSSU matrix included 71 sequences (802 bp alignment length), with a nucleotide diversity of 0.019; and the RPB1 data set was comprised of 53 sequences (537 bp), with a nucleotide diversity of 0.020. The concatenated DNA matrix is available online (Available in TreeBase Submision ID-18070). Individual gene topologies didn't showed well-support topological incongruence. Phylogenies inferred from the multilocus, concatenated datasets using ML and Bayesian inference had a similar topology enabling the joint representation (Fig 1). A single difference between the RaxML andMr.Bayes topologies was the position of three samples "*P. hypoleucites*_Kenya", "*P. bolliana*_USA" and "*P. appalachiensis*_USA".

Five major, well-supported clades can be distinguished in the *Punctelia* phylogeney (indicated by the letters A-E). Clade 'A'–the P. rudecta group–was comprised of a well-supported clade with samples of *P. rudecta* s. lat. from the Canary Islands (hereinafter *P. guanchica*), which is sister to a clade that consists of North American *P. rudecta* samples (hereinafter *P. rudecta* s. stricto), a single specimen identified as *P. hypoleucites* downloaded from Genbank, and also a specimen from South Africa identified as *P. toxodes* that is sister to

the rest of the group. Clade ‘B’ consists of two sister clades. The first is comprised of samples

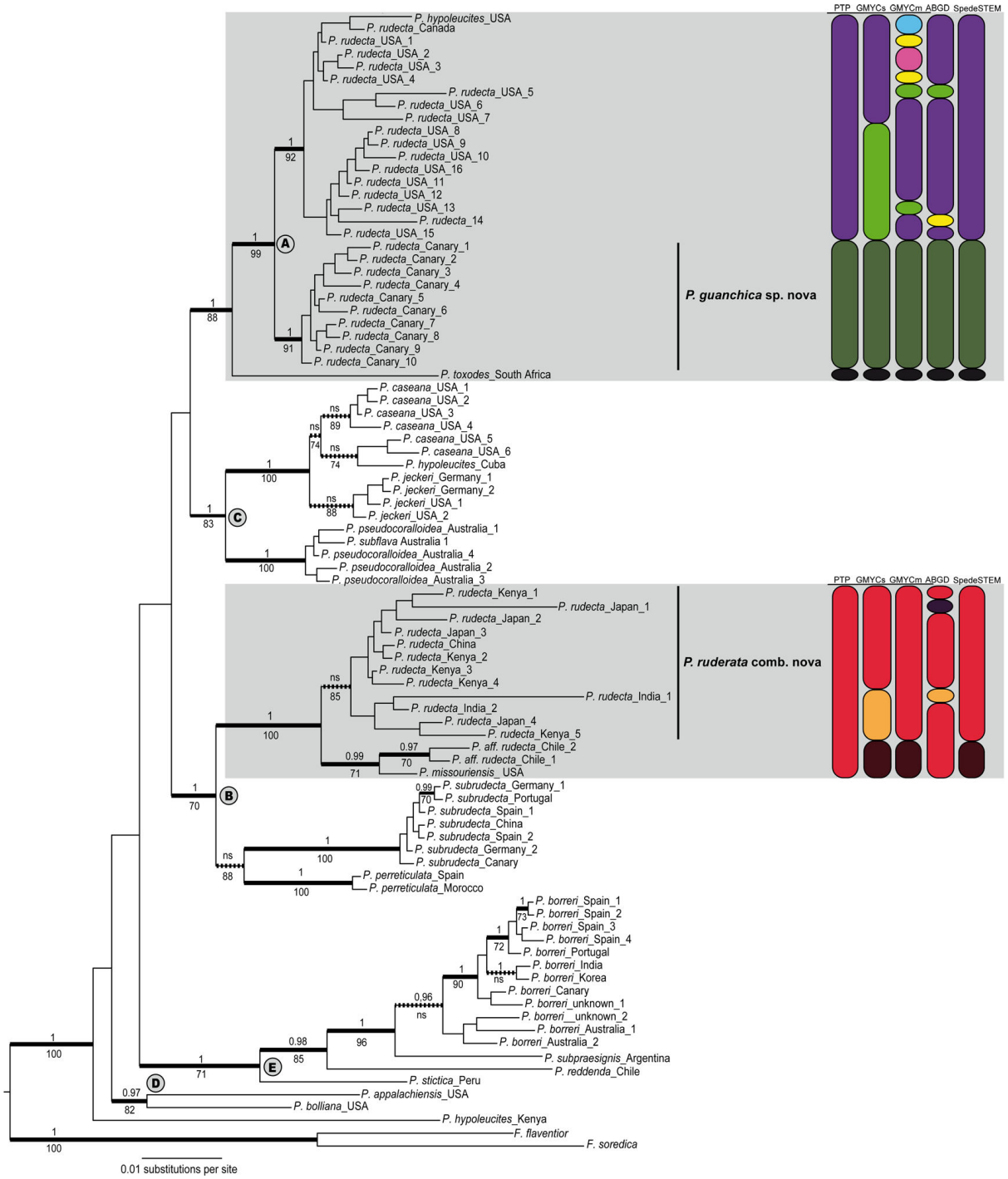

Fig 1. Concatenated gene tree topology. Gene tree of *Punctelia genus*, focused on *Punctelia rudecta* s. lat. values on branches represents bootstrap support (bs) equal or higher than 70 and posterior probability (pp) equal or higher than 0.95. Wider bolded lines correspond to branches supported by both bs and pp, dotted lines represent branches supported by bs or pp. Species delimitation scenarios resulted from different methods indicated in columns to the right (details discussed in text). ns = not supported.

identified as *P. rudecta* s. lat. from Asia and East Africa (hereinafter *P. ruderata*), and *P. missouriensis*, which forms a sister-group to two *P. aff. rudecta* samples from Chile. The other group within clade 'B' includes two samples of *P. perreticulata* and seven samples of *P. subrudecta*. Both these species form well-supported monophyletic lineages. Clade 'C' includes

two well-supported clades, one with samples representing *P. caseana* and *P. jeckeri* (each forming monophyletic groups, but only the latter being strongly supported), whereas the other clade includes samples from Australia that were identified as *P. pseudocoralloidea* and *P. subflava*. The species of clades 'A'-'C' are all characterized by the presence of lecanoric acid as major medullary component and forms a well-supported clade in trees performed in *Beast species tree (Fig 2). Clade 'D' includes samples of two species containing fatty acids as major medullary compounds, *P. appalachensis* and *P. bolliana*. Clade 'E' includes species containing gyrophoric acid as main compound and is comprised of samples representing *P. borreri*, forming a well-supported monophyletic group, nested to *P. subpraesignis*, *P. reddenda*, and *P. stictica*. The relationship of *P. hypoleucites* from Kenya is uncertain and was recovered as basal to all the clades (Fig 1).

Punctelia rudecta as currently circumscribed was recovered as polyphyletic with samples forming distinct lineages within clades 'A' and 'B' (Fig 2), with each lineages restricted to distinct geographic regions. Chilean samples *P. aff. rudecta* s. formed a monophyletic group within clade 'B', specimens representing *P. ruderata* from Africa and Asia formed another monophyletic group within clade 'B', and North American material representing *P. rudecta* s. str. were restricted to a strongly supported group within clade 'A'. Finally, *all P. guanchica* specimens from the Canary Islands formed a sister-group to the North American specimens within clade 'A'. A single collection representing *P. toxodes* from South Africa was sister to all remaining samples within clade 'A'. Alternative hypothesis tests rejected monophyly of *P. rudecta* under both tests (SH and ELH); however, only 'constraint 2' strongly rejects monophyly of *P. rudecta* s. lat. ($p<0.001$ in SH and ELW tests) (Table 1).

Table 1. Alternative hypothesis testing

Alternative hypothesis testing	SH p-value	ELH p-value
Constrain 1	0.047*	0.0477*
Constrain 2	<0.001***	<0.001***
Asterisk symbols represents the level of statistical significance. *, p value equal or less tan 0.05. ***, p value equal or less tan 0.001		

Species delimitation analyses The sampling for the species delimitation analyses was only sufficient for *P. rudecta* s. lat. and hence all other *Punctelia* species included in this study are assumed to represent the identified nominal taxa. Speciation probabilities estimated in BP&P shown high posterior probability for most of the candidate species in the *Punctelia rudecta* complex, including: *P. guanchica*, *P. rudecta* s. stricto, *P. toxodes* and *P. ruderata* (Fig 2). However, the isidiate samples identified as *P. rudecta* from Chile or the sorediate *P. missouriensis* were not supported with high speciation probabilities (Fig 2).

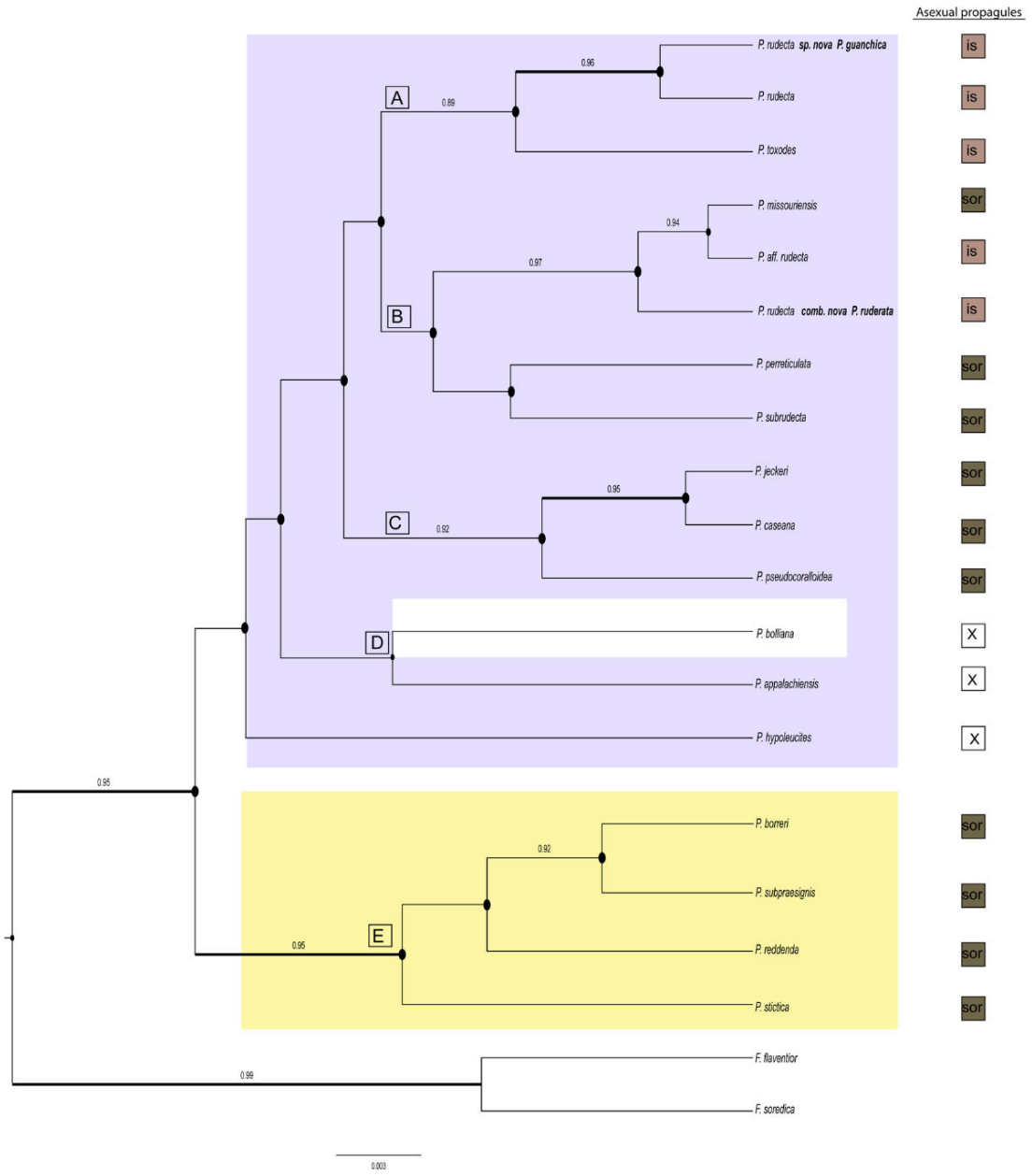

Fig 2. Coalescent-based species tree of the genus *Punctelia*. This is a bayesian species tree inferred in *Beast using three loci (mtSSU, ITS, RPB1). Posterior probabilities are represented by numbers above the branches and nodal support calculated in BP&P with algorithm 1 is indicated with circles on the nodes. Coloured squares represent chemical compounds; species containing gyrophoric acid are shown in yellow and species containing lecanoric acid in gery colours. Abbreviations on the right column are: is = isidiate, sor = sorediate and X = propagules absent.

The species tree inferred using *Beast showed high PP for the sorediate species included in the A-B clade *P. perreticulata* and *P. subrudecta* included in clade B. For *P. rudecta* specimens recovered in clade 'A', the additional analyses (ABGD, $GMYC_{single}$, $GMYC_{multiple}$, PTP, spedeSTEM) supported the clade consisting of *P. guanchica* samples from the Canary Islands as a distinct, species-level lineage (Fig 1, Fig 2). The results for its sister-group, *P. rudecta* sensu stricto from North America is a well supported lineage but is recognized by $GMYC_{single}$ as two different lineages, while ABGD and $GMYC_{multiple}$, recognized additional putative lineages within this group (Fig 1). The South African sample recognized as *P. toxodes*, was identified as a distinct lineage in all six analyses. In clade 'B', *P. ruderata*, *P. aff. rudecta* and *P. missouriensis* were included into a single lineage by PTP, while $GMYC_{single}$, $GMYC_{multiple}$ and spedeSTEM recognized one lineage composed by *P. missouriensis* and *P. aff. rudecta* differentiated from *P. ruderata*, whereas ABGD identified multiple lineages (Fig 1).

Phenotypical re-examination of *Punctelia rudecta* samples In order to phenotypically characterize putative species-level lineages found in our sampling of isidiate specimens in clades 'A' and 'B' (*P. rudecta* sampling s. lat.), we studied morphological characters and secondary chemistry of the samples included in the analyses. Morphological characters, such as thallus color, presence and form of isidia, presence of ascomata, and conidiomata, showed some variability but only few of these characters were associated with the clades found in the phylogenetic analyses (Supplementary Table 1). Thallus color was variable within putative lineages, and failed to consistently diagnose species-level lineages. The lower surface was usually pale brown, but also ranging into a darker brown coloration in some specimens. The upper surface showed a gradation from brownish green to bluish grey. To ensure any result about the importance of color, only recently collected specimens were considered because the color tonality is gradually lost after collection. Isidia morphology showed some variability even among lobes within the same thallus. In order to systematize the isidia form comparison, the degree of ramification was taken in account, and two times dichotomous ramificated isidia were most commonly observed. When the isidia were three or more times dichotomously branched, they took coralloid or dendroid appearance. The specimen representing *P. toxodes* was the only collection showing unbranched isidia. Despite the

inclusion of a number of collections from the Canary Islands, we failed to find ascomata or conidiomata on them, whereas specimens from East Africa, Asia or South America frequently have mature ascomata. In North American samples, young immature ascomata were occasionally found. Ascospores comparison between groups was not possible, and a comparison of conidiospore size failed to distinguish distinct lineages. It is interesting to note that in *P. ruderata* and *P. aff. rudecta* from Chile conidiomata and ascomata were found within the same thallus usually however these are not found in the single thallus of the sample of *P. rudecta* or *P. toxodes*. While all specimens of *P. rudecta* s. lat. contained atranorin and lecanoric acid as major compounds, the samples from Chile, Kenya, Japan and India often also contained unidentified fatty acids, which were not found in the other specimens examined. While most *P. rudecta* s. lat. species occur mainly on bark, *P. guanchica* sp. nov. differs in substrate preference, occurring mainly rocks and occasionally the base of a shrub. We have confirmation that the species *P. toxodes* also occurs on rock (personal observation).

Discussion

Phylogenetic analyses and species delimitation Inferences from previous phylogenetic studies on *Punctelia* have been limited by relatively small sample sizes and the use of only one or two molecular markers (Crespo *et al.* 2004; Lendemer & Hodkinson 2010). In this study, five major, well-supported clades were found in *Punctelia* (Fig 1), although relationships among these clades were partially known (Fig 2). Interestingly the major clades in *Punctelia* are characterized by their medullary chemistry. Clades 'A', 'B', and 'C' are comprised of specimens producing the depside lecanoric acid, whereas species in clade 'D' produce the related tridepside gyrophoric acid. Clade 'E' is characterized the presence of fatty acids. Secondary chemistry has been found previously in other, only distantly related groups of lichenized fungi to be a predictor of phylogenetic relationships, including *Baeomycetaceae* (Platt & Spatafora 2000), *Graphidaceae* (Lumbsch *et al.* 2014), *Pertusariaceae* (Schmitt & Lumbsch 2004; Lumbsch *et al.* 2006), *Teloschistaceae* (Arup *et al.* 2013), and also several clades within *Parmeliaceae* (Crespo *et al.* 2010; Crespo *et al.* 2011; Myllys *et al.* 2011).

Punctelia rudecta as currently circumscribed has been shown to be polyphyletic with samples falling into five distinct clades. The BP&P analysis showed species with high statistical

support in the isidiate species complex belonging to clades 'A' and 'B' (Fig 2) recognizing four species; *P. rudecta*, *P. guanchica* sp. nov., *P. toxodes*, and *P. ruderata*. Nodal support for the *P. guanchica P. rudecta* clade 1, the node separating *P. toxodes* from the previous node 1, the node which separates *P. ruderata* from *P. missouriensis*, and *P. aff. rudecta* were all estimated at PP = 0.99 (Fig 2). The node separating *P. missouriensis* and *P. aff rudecta* was the only split that didn't showed strong statistical support, and it appears that this uncertainty most probably results from the fact that these lineages was represented only by ITS sequence data (Supplementary Table 1).

Species delimitation analyses based on additional analyses supported between four and ten candidate species in *P. rudecta* s. lat. (Fig 1), with PTP identifying the lowest (4 OTUs) and $GMYC_{multiple}$ the highest number of species (10 OTUs). The latter method has been suggested to overestimate the number of clusters under certain conditions (Esselstyn *et al.* 2012; Fujisawa & Barraclough 2013). With the data at hand, we follow a conservative approach as advocated by Miralles and Vences (Miralles & Vences 2013) who argued that it would be better to fail to delimit species to falsely delimit entities that do not represent independent lineages. The results of PTP, spedeSTEM and BPP were consistent in circumscribing four species in both the PTP and BP&P analysis, plus a fifth lineage supported in the spedeSTEM analysis which included the samples *P. missouriensis* and *P. aff. rudecta* (Fig 1, Fig 2). The taxonomic conclusions to accommodate the four species identified under the name *Punctelia rudecta* are drawn in the section below.

This study adds another example to the growing number of cases reviewed (Crespo & Perez-Ortega 2009; Crespo & Lumbsch 2010; Lumbsch & Leavitt 2011; Leavitt *et al.* 2015) in which species that were thought to have wide distributional ranges have subsequently been split into distinct cryptic lineages with more restricted distributional ranges. Morphological and chemical reexamination of the samples did not reveal phenotypical characters that would allow identification without having molecular data at hand, except the presence of unidentified fatty acids in *P. ruderata* and *P. aff. rudecta*, and smaller and unbranched isidia in the South African material named *Punctelia toxodes*. This name has recently been resurrected based on morphological features, including dorsiventrally flattened isidia and unciform, cylindrical, curved conidia (Kalb 2007). In our samples the isidia are shorter, unbranched, thick and rarely flattened

with age. Since we only had two specimens from South Africa available for study and were unable to assess the variation of these characters within the population. Isidial morphology is very variable in *Punctelia rudecta* sensu lato and in lichenized fungi in general and thus it could not be considered as a useful feature to discriminate species. Ecological traits can be useful to discriminate *P. guanchica* sp. nov. which grows on volcanic rocks, while our samples of *P. rudecta* s. stricto, *P. aff. rudecta* and *P. ruderata* were exclusively corticolous. The taxon *P. toxodes* is known to grow on both rocks and tree bark. The identification of specimens will not cause issues for field biologists, since the species that are here recognized are geographically isolated. Geographical structure of species identified using molecular data has been recently shown to be a common phenomenon in lichenized fungi (Argüello *et al.* 2007; Otálora *et al.* 2010; Amo de Paz *et al.* 2012; Parmmen *et al.* 2012; Moncada *et al.* 2014).

Here we use an integrative taxonomic approach to circumscribe and formally recognize species in *P. rudecta* s. lat. Integrative taxonomy uses independent sources of data, such as molecular, morphological, ecological, and/or geographical data to delimit species (Schlick-Steiner *et al.* 2010). However, these data sources are limited especially for lichenized and non-lichenized fungi and collecting these could be a great challenge for cryptic lineages. In these cases, coalescent-based approaches have been widely used as an objective measure for identifying divergent evolutionary lineages (Fujita *et al.* 2012; Carstens *et al.* 2013). Nonetheless, identifying geographical/and or ecological differences between lineages could also help to understand speciation process. Our results show that implementing a variety of species delimitation methods, coupled with assessment of other data types as in this case geography could play an important role in identification of species and speciation process in lichenized fungi.

Taxonomic conclusions As discussed above, we propose the formal recognition of four species in the *Punctelia rudecta* s. lat: *P. rudecta* s. str., *P. ruderata, P. toxodes*, and the newly described *P. guanchica* sp. nov. The type specimen of *P. rudecta was* collected in North America (Hale 1965; Krog & Swinscow 1977), and hence this name should be used for the North American clade (epitype: USA, North Carolina, Swain County, Great Smoky Mountains National Park, Beauregard Ridge, 35°27’38” N, 83°26’17” W, alt. 560m, On Acer, 14th Oct. 2010,

J.C. Lendemer 26959 [MAF-Lich 19163; Ref. Sequences KR024454 (ITS), KR024500 (mtSSU), KR024541 (RPB1)], selected here). The name *Punctelia toxodes* (Stirt.) Kalb & Götz. (Bas.: *Parmelia toxodes* Stirt., Scot. Nat. 4: 253 [1877–78]; epitype: South Africa, Cape region, Paarl Mountain, 33°44'26" S, 18°56'55" E, alt. 530m, on tree trunk and twigs, 30th May 2005, A. Crespo, P.K. Divakar, D.L. Hawksworth, G. Amo & H.T. Lumbsch [MAF-Lich 19757; Ref. Sequences KR024412 (ITS), KR024460 (mtSSU), KR024504 (RPB1)]), selected here) is available for the South African clade. The name *Punctelia ruderata* (Vainio) Canêz & Marcelli was resurrected in a Ph.D. thesis (Canêz 2009) and reported from Brazil. However, the combination has not been validly published (Art 29.1, ICBN). Further, our results indicate that *P. ruderata* in our circumscription does not occur in Brazil. Therefore, for the Old world clade identified in this study, we propose to resurrect the name *Punctelia ruderata* (Vain.) Canêz & Marcelli ex. Alors *et al.* comb nov. (Index Fungorum no. IF 551455; Bas.: *Parmelia ruderata* Vain., Bot. Mag. Tokyo 35: 47 [1921]; epitype: Japan, Honshu, Musashi (Pref. Saitama), Takizawa Dam, Ohtaki, Chichibu city, 35° 56' 31" N 138° 53' 58" E alt. 577m, on *Prunus yedoensis*, 7th Feb. 2009, A. Crespo, P.K. Divakar & Y. Ohmura 6018w [MAF-Lich 18877; Ref. Sequences KR024434 (ITS), KR024521 (RPB1)]), selected here). There is no available name for the Canary Islands clade and hence we describe a new species for this clade below.

***Punctelia guanchica* Alors, A. Crespo & Divakar, sp. nov.**

Index Fungorum: IF551458

Diagnosis: Morphologically similar to *P. rudecta* sensu stricto, but differs in having thicker isidia developing from the centre of pseudocyphellae, saxicolous habitat, and DNA characters; only known from the Canary Islands.

Type: Spain: Canary Islands, Tenerife, La laguna, Las Mercedes, monte verde, 28°32' 41" N 16°17'49" W, alt. 613m, on vertical basaltic rock, 20th June 2009, A. Crespo, P. Cubas, A. Santos & P. K. Divakar, 6046v (MAF-Lich 18871−holotype; F−isotype).

Description: Thallus foliose, adpressed to the substratum, 3–4 cm across; lobes rotund, 2–4 mm wide, margins entire, eciliate. Upper surface whitish grey, often bordered by a narrow, brown margin, reticulately rugulose near the margin of lobes, pseudocyphellate and isidiate. Pseudocyphellae laminal, punctiform to elongate, up to 0.5 mm in size, more distinct near peripheral zone. Isidia laminal, developing from the centre of pseudocyphellae, thick, short,

up to 0.5 mm tall, simple to coralloid branched, in groups, rarely flattened in the centre of thallus (image of *P. guanchica* at the chapter title page). Medulla white. Lower surface white, with pale margin, rhizines simple, concolours with the lower surface, ca. 1 mm long. Photobiont trebouxioid. Apothecia and conidia unknown.

Chemistry: Cortex K+ yellow; medulla K-, C+ rose, KC+ red, PD-; atranorin and lecanoric acid. Reference sequences: GenBank accession numbers. KR024415 (ITS), KR024463 (mtSSU) and KR024506 (RPB1) Additional specimens examined: The samples are listed in Supplementary Table 1.

Etymology: The epithet 'guanchica' refers to the guanches, the aboriginal inhabitants of the Canary Islands, since the species is only known from this archipelago.

Remarks: In the field the new species can easily be confused with *P. rudecta* sensu stricto, which occurs in North America and differs in having isidia developing from the periphery of pseudocyphellae, and is mainly corticolous. *P. guanchica* is only known from Canary Islands and has only been found on saxicolous habitats. The new species is also similar to *P. toxodes* that is endemic to South Africa. Morphologically *P. guanchica* is also similar to *P*. *ruderata*, which occurs in Asia and East Africa, and belongs to an independent, supported monophyletic clade (clade B, Fig 1 Fig 2).

Author Contributions Conceived and designed the experiments: DA HTL PKD SDL AC. Performed the experiments: DA PKD. Analyzed the data: DA HTL PKD SDL. Contributed reagents/materials/analysis tools: DA HTL PKD SDL AC. Wrote the paper: DA HTL PKD SDL AC.

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Suplementary Material

Supplementary Table 1 GenBank accession numbers for the three sampled loci: nuclear ribosomal internal transcribed spacer region (ITS), mitochondrial small subunit (mtSSU), and protein-coding maker RPB1. Newly generated sequences for this study are indicated in bold. Type specimens are indicated with asterisk (*).

Species	Voucher	Locality	GenBank accession numbers		
			ITS	mtSSU	RPB1
Flavopunctelia soredica	Maf-Lich 17771	USA, Minesota	Divakar *et al.* 2015	Divakar *et al.* 2015	GU994700
Flavopuntelia flaventior	Maf-Lich 6046	Spain, Teruel	AY581060	AF351164	EF092110
P. aff. rudecta Chile 1	Maf-Lich 19170	Chile, Mendoza	**KR024424**	xxx	xxx
P. aff. rudecta Chile 2	Maf-Lich 19173	Chile, Mendoza	**KR024458**	xxx	xxx
P. appalachiensis USA	Maf-Lich 19154	USA, Tennessee	**KR024455**	**KR024501**	**KR024542**
P. bolliana USA	Maf-Lich 17774	USA, Minnesota	GU994579	GU994673	GU994733
P. borreri Australia 1	Maf-Lich 19186	Australia, Victoria	**KR024449**	**KR024495**	**KR024536**
P. borreri Australia 2	Maf-Lich 19187	Australia, Victoria	**KR024450**	**KR024496**	**KR024537**
P. borreri Canary	Maf-Lich 10246	Canary Islands, Tenerife	AY613400	AY613422	xxx
P. borreri India	Maf-Lich 16125	India, Uttaranchal	HM016964	xxx	xxx
P. borreri Portugal	Maf-Lich 10237	Portugal, Coimbra	AY613401	AY613424	xxx
P. borreri S. Korea	Hur 030736	South Korea	DQ394373	xxx	xxx
P. borreri Spain 1	Maf-Lich 10238	Spain, Castellon	AY613399	AY613423	xxx
P. borreri Spain 2	Maf-Lich 10240	Spain, Madrid	AY613409	AY613429	xxx
P. borreri Spain 3	MafLich10254	Spain, Pontevedra	AY613404	AY613421	xxx
P. borreri Spain 4	MafLich10255	Spain, Arcos (Cádiz)	AY613405	AY613425	xxx
P. borreri unknown 1	Hur J080105	unknown	GU593035	xxx	xxx
P. borreri unknown 2	Hur TW090011	unknown	GU593038	xxx	xxx
P. caseana USA 1	Maf-Lich 19184	USA, Ohio	**KR024445**	**KR024491**	**KR024532**
P. caseana USA 2	Maf-Lich 19176	USA, Ohio	**KR024446**	**KR024492**	**KR024533**
P. caseana USA 3	Maf-Lich 19180	USA, Ohio	**KR024447**	**KR024493**	**KR024534**
P. caseana USA 4	Lendemer 15041	USA, New Jersey	GU384881	xxx	xxx
P. caseana USA 5	Lendemer 13428	USA, Pennsylvania	GU384886	xxx	xxx
P. caseana USA 6	Lendemer 12733	USA, Pennsylvania	GU384879	xxx	xxx
P. guanchica Canary 1	Maf-Lich 18870	Canary Islands, Tenerife	**KR024425**	**KR024472**	**KR024514**

P. guanchica Canary 2	Maf-Lich 18873	Canary Islands, Tenerife	**KR024427**	**KR024474**	xxx
P. guanchica Canary 3	Maf-Lich 19166	Canary Islands, La Palma	**KR024430**	**KR024477**	**KR024517**
P. guanchica Canary 4	Maf-Lich 18866	Canary Islands, La Palma	**KR024429**	**KR024476**	**KR024516**
P. guanchica Canary 5*	Maf-Lich 18871*	Canary Islands, Tenerife	**KR024415**	**KR024463**	**KR024506**
P. guanchica Canary 6	Maf-Lich 19165	Canary Islands, Tenerife	**KR024416**	**KR024464**	**KR024507**
P. guanchica Canary 7	Maf-Lich 19153	Canary Islands, Tenerife	**KR024414**	**KR024462**	**KR024505**
P. guanchica Canary 8	Maf-Lich 18868	Canary Islands, Tenerife	**KR024426**	**KR024473**	xxx
P. guanchica Canary 9	Maf-Lich 19167	Canary Islands, La Palma	**KR024428**	**KR024475**	**KR024515**
P. guanchica Canary 10	Maf-Lich 10256	Canary Islands, Tenerife	AY613403	AY613420	xxx
P. hypoleucites Cuba	Maf-Lich 19168	Cuba, Guantanamo	**KR024459**	**KR024503**	**KR024545**
P. hypoleucites Kenya	Maf-Lich 19168	Kenya, Western Province	**KR024413**	**KR024461**	xxx
P. hypoleucites USA	DUKE 12987 (02.06.2002)	USA, Texas	HQ650685	AY584629	DQ912364
P. jeckeri Germany 1	Maf-Lich 10249	Germany, Dusseldorf	AY613407	AY613427	xxx
P. jeckeri Germany 2	Maf-Lich 10251	Germany, Dusseldorf	AY613406	AY613426	GU994731
P. jeckeri USA 1	McCune 29576	USA, Oregon	GU384891	xxx	xxx
P. jeckeri USA 2	Lendemer 14739	USA, California	GU384890	xxx	xxx
P. misouriensis USA	Lendemer 15006	USA, Ohio	GU384892	xxx	xxx
P. perreticulata Morocco	Maf-Lich 16140	Morocco, Medium Atlas	HM016967	xxx	xxx
P. perreticulata Spain	Maf-Lich 10239	Spain, Castellon	AY613391	AY613411	xxx
P.pseudocoralloidea Aus 1	Maf-Lich 6922	Australia, New South Wales	AY586572	AY586595	EF092150
P.pseudocoralloidea Aus 2	Maf-Lich 19188	Australia, Victoria	**KR024452**	**KR024498**	**KR024539**
P.pseudocoralloidea Aus 3	Maf-Lich 18872	Australia, Victoria	**KR024453**	**KR024499**	**KR024540**
P. seudocoralloidea Aus 4	Maf-Lich 19189	Australia, Victoria	**KR024451**	**KR024497**	**KR024538**
P. reddenda Chile	Maf-Lich 10247	Chile, Valdivia	AY613410	AY613430	GU994732
P. rudecta Canada	Normore 7385	Canada	Divakar *et al.* 2015	GU994672	xxx
P. rudecta USA 1	Maf-Lich 18254	USA, New Hampshire	**KR024436**	**KR024482**	**KR024523**
P. rudecta USA 2	Maf-Lich 18259	USA, New Hampshire	**KR024438**	**KR024484**	**KR024525**
P. rudecta USA 3	Maf-Lich 18256	USA, New Hampshire	**KR024439**	**KR024485**	**KR024526**
P. rudecta USA 4	Maf-Lich 18255	USA, New Hampshire	**KR024437**	**KR024483**	**KR024524**
P. rudecta USA 5	Maf-Lich 19177	USA, Ohio	**KR024443**	**KR024489**	**KR024530**
P. rudecta USA 6 *	Maf-Lich 19163 *	USA, North Carolina	**KR024454**	**KR024500**	**KR024541**
P. rudecta USA 7	Maf-Lich 19160	USA, Maine	**KR024457**	xxx	**KR024544**
P. rudecta USA 8	Maf-Lich 19175	USA, Ohio	**KR024442**	**KR024488**	**KR024529**

P. rudecta USA 9	Maf-Lich 19182	USA, Ohio	**KR024444**	**KR024490**	**KR024531**
P. rudecta USA 10	Maf-Lich 19178	USA, Ohio	**KR024448**	**KR024494**	**KR024535**
P. rudecta USA 11	Maf-Lich 19181	USA, Ohio	**KR024441**	**KR024487**	**KR024528**
P. rudecta USA 12	Maf-Lich 19183	USA, Ohio	**KR024440**	**KR024486**	**KR024527**
P. rudecta USA 13	Maf-Lich 18260	USA, New Jersey	**KR024417**	**KR024465**	**KR024508**
P. rudecta USA 14	Maf-Lich 19159	USA, Missouri	**KR024456**	**KR024502**	**KR024543**
P. rudecta USA 15	DUKE 5520 (01-26-03.7)	USA, North Carolina	HQ650686	AY584630	DQ912365
P. rudecta USA 16	Maf-Lich 7661	USA, Mayrland	AY586573	AY586596	xxx
P. ruderata China	Maf-Lich 10253	China, Yunan	AY613402	AY613419	xxx
P. ruderata India 1	Maf-Lich 18262	India, Tamil Nadu	**KR024431**	**KR024478**	**KR024518**
P. ruderata India 2	Maf-Lich 18263	India, Tamil Nadu	**KR024432**	**KR024479**	**KR024519**
P. ruderata Japan 1	Maf-Lich 18878	Japan, Ibaraki	**KR024433**	**KR024480**	**KR024520**
P. ruderata Japan 2	Maf-Lich 18874	Japan, Saitama	**KR024422**	**KR024470**	xxx
P. ruderata Japan 3	Maf-Lich 18875	Japan, Ibaraki	**KR024423**	**KR024471**	**KR024513**
P. ruderata Japan 4 *	Maf-Lich 18877*	Japan, Saitama	**KR024434**	xxx	**KR024521**
P. ruderata Kenya 1	Maf-Lich 18270	Kenya, Thompson Falls	**KR024420**	**KR024468**	**KR024511**
P. ruderata Kenya 2	Maf-Lich 18268	Kenya, Thompson Falls	**KR024419**	**KR024467**	**KR024510**
P. ruderata Kenya 3	Maf-Lich 18269	Kenya, Thompson Falls	**KR024418**	**KR024466**	**KR024509**
P. ruderata Kenya 4	Maf-Lich 18267	Kenya, Thompson Falls	**KR024421**	**KR024469**	**KR024512**
P. ruderata Kenya 5	Maf-Lich 18271	Kenya, Ngong	**KR024435**	**KR024481**	**KR024522**
P. stictica Peru	DNA 1609 (HBG)	Peru	AY773119	xxx	xxx
P. subflava Australia 1	Elix 42705	Australia	AY586575	AF351183	xxx
P. subpraesignis Argentina	DNA 1310 (HBG, LD)	Argentina, Buenos Aires	AY267010	xxx	xxx
P. subrudecta Canary	Maf-Lich 10242	Canary Islands, Tenerife	AY613397	AY613417	xxx
P. subrudecta China	Maf-Lich 10244	China, Yunnan	AY613395	AY613413	xxx
P. subrudecta Germany *1*	Maf-Lich 10243	Germany, Northrhine-Westfalia	AY613393	AY613414	xxx
P. subrudecta Germany 2	Maf-Lich 10250	Germany, Dusseldorf	AY613394	AY613416	xxx
P. subrudecta Portugal	Maf-Lich 9918	Portugal, Nazaré	AY581089	AY582325	Divakar *et al.* 2015
P. subrudecta Spain 1	Maf-Lich 10241	Spain, Pontevedra	AY613392	AY613412	xxx
P. subrudecta Spain 2	Maf-Lich 10245	Spain, Madrid	AY613396	AY613416	xxx
P. toxodes South Africa *	Maf-Lich 19757 *	South Africa, Cape Region	**KR024412**	**KR024460**	**KR024504**

Supplementary Table 2 Table summarizing information obtained from the vouchers of *P. rudecta* s. lat. samples, including size, presence of sexual structures, coloration, chemistry and substrate

Specie	Label	Substrate	Isidia	Colour up/down	Fatty acids	Apothecia	Conidia	Conidiospores shape & size
P. aff. rudecta	Chile 1	tree	dicotomous	green/brown	yes	yes	yes	
P. aff. rudecta	Chile 2	tree	dicotomous	greenish/brown	yes	yes	yes	unciform, 7.2 µm
P. guanchica	Canary1	rock	dicotomous	greenish/pale	no	no	no	
P. guanchica	Canary2	rock	dicotomous	grey/pale brown	no	no	no	
P. guanchica	Canary3	rock	dicotomous	greenish/pale	no	no	no	
P. guanchica	Canary4	rock	dicotomous	greenish/pale	no	no	no	
P. guanchica	Canary5	rock	dendritic	brownish green/pale	no	no	no	
P. guanchica	Canary6	shrub	dicotomous	greenish/pale	no	no	no	
P. guanchica	Canary8	rock	dicotomous	greenish/brown	no	no	no	
P. guanchica	Canary9	rock	dicotomous	greenish/pale	no	no	no	
P. rudecta	USA 1	tree	dendritic	greenish/pale	no	no	yes	
P. rudecta	USA 2	tree	dicotomous	greenish/pale brown	no	no	yes	hooked, 7.3 µm
P. rudecta	USA 3	tree	dendritic	greenish/pale	no	no	no	
P. rudecta	USA 4	tree	dicotomous	greenish/pale	no	yes	no	
P. rudecta	USA 5	tree	dendritic	greenish/pale	no	yes	yes	
P. rudecta	USA 6	tree	dendritic	greenish/pale	no	yes	yes	
P. rudecta	USA 7	tree	dicotomous	dark green/pale	no	no	yes	hooked - unciform, 7.2 µm
P. rudecta	USA8	tree	dendritic	greenish/pale	no	no	yes	hooked, 7.4 µm
P. rudecta	USA9	tree	dendritic	bluish gray/pale	no	yes	no	
P. rudecta	USA10	tree	dendritic	bluish gray/pale	no	no	no	
P. rudecta	USA11	tree	dendritic	greenish/pale	no	yes	yes	
P. rudecta	USA12	tree	dicotomous	greenish/pale	no	no	no	
P. rudecta	USA13	tree	dicotomous	white/pale	no	no	no	
P. rudecta	USA14	tree	dicotomous	blueish green/pale	no	no	no	
P. ruderata	India 1	tree	dendritic	green/pale	yes	no	no	
P. ruderata	India 2	tree	dicotomous	dark green/brown	yes	no	no	
P. ruderata	Japan 1	tree	dicotomous	brownish green/pale	yes	no	yes	
P. ruderata	Japan 2	tree	dendritic	brownish green/pale	yes	yes	no	
P. ruderata	Japan 3	tree	dicotomous	yellowish green/pale	yes	no	yes	unciform, 7.1 µm
P. ruderata	Japan 4	tree	dendritic	yellowish green/pale	yes	yes	no	
P. ruderata	Kenya 1	tree	branched	greenish/brown	yes	no	yes	
P. ruderata	Kenya 2	tree	dicotomous	yellowish green	yes	yes	no	
P. ruderata	Kenya 3	tree	dendritic	brownish green/pale	yes	yes	no	
P. ruderata	Kenya 4	tree	dendritic	dark green/pale	yes	yes	no	
P. ruderata	Kenya 5	tree	dendritic	greyish green/pale	yes	no	yes	unciform, 7 µm
P. toxodes	S. Africa	tree	not ramificated	brownish green/brown	no	yes	yes	bifusiform - unciform, 8 µm

CAPITULO II / CHAPTER II

Insights into intrathalline genetic diversity of the Cosmopolitan lichen symbiotic green alga *Trebouxia decolorans* Ahmadjian using microsatellite markers

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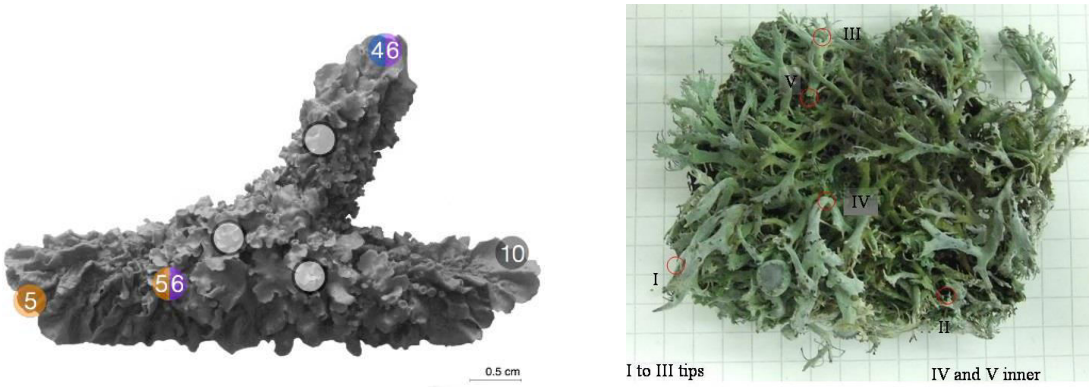

Xanthoria parietina thallus (left*) and Anaptychia ciliaris* thallus (right) cercles represents exact location where within thalli samples were taken.

Abstract

Trebouxia decolorans is a widespread and common symbiotic green alga that is found in association with different species of lichen-forming fungi. By applying *T. decolorans*-specific microsatellite markers, we investigated the within-thallus diversity of *T. decolorans* in thalli of *Xanthoria parietina* and *Anaptychia ciliaris*. We found several algal strains in most of the thalli of both hosts. High genetic differentiation among thalli suggests that algal diversity is generated de novo via mutation in both fungal hosts. Rarefied allelic richness of the algae was higher in thalli of *X. parietina*. Our results indicate that in *X. parietina* intrathalline algal diversity is additionally created by environmental uptake of algae either at the start of the symbiotic association or during the lifetime of the thallus. This study indicates that promiscuous host-symbiont associations in lichen symbioses with *Trebouxia spp*. may be more common than currently recognized.

Keywords: Trebouxia, *Xanthoria parietina*, *Anaptychia ciliaris*, Microsatellite, Population, Symbiosis

Introduction

Progress in the molecular techniques that resolve microbial diversity ushers a new phase in symbiosis research. Recent studies show that microbial symbiotic partners often comprise genetic mosaics rather than homogeneous populations (Loxdale & Lushai 2003; Pineda-Krch & Lehtilä 2004). Examples include coral-dinoflagellate symbioses (Andras *et al.* 2011; Pettay *et al.* 2011; Howells *et al.* 2013), insect-bacteria (Burkholderia or Wolbachia) symbioses (Russell *et al.* 2009; Kikuchi *et al.* 2011), plant-bacteria (*Rhizobium* or *Frankia*) symbioses (Yamanaka *et al.* 2003; Kiers & Denison 2008), and ant-plant (*Pseudomyrmex* – *Acacia*) symbioses (Kautz *et al.* 2009). The presence of multiple symbiont genotypes suggests that symbionts can be acquired from the environment and may be important in local adaptation of symbiotic organisms (Byler *et al.* 2013). For example, the high diversity of chemosynthetic bacteria in a gutless oligochaete was found to provide multiple sources of carbon to the host (Dubilier *et al.* 2008). LaJeunesse *et al. (*2010) showed that corals can host different locally adapted strains of *Symbiodinium* to broaden the range of physiological responses when subjected to selective processes such as climate change.

Lichen-forming fungi, their photosynthetic partners (green algae and/or cyanobacteria), and specific communities of non-photosynthetic bacteria form a symbiotic system that can be regarded as a self-contained miniature ecosystem (Grube *et al.* 2009; Hodkinson *et al.* 2012). Sequence-based studies and DGGE fingerprinting showed a wide range of association scenarios, from host specialization where the mycobiont accepts only a single algal strain, to generalism where the host accepts multiple photobionts (Beck *et al.* 1998; Blaha *et al.* 2006; Muggia *et al.* 2013). Some species of lichen-forming fungi are promiscuous with multiple algal lineages co-inhabiting a single lichen thallus, e.g. *Evernia mesomorpha*, *Ramalina farinacea*, *Rinodina atrocinerea*, *R. tunicata*, and *Protoparmeliopsis muralis* (Casano *et al.* 2011; Helms *et al.* 2001; Muggia *et al.* 2013; Piercey-Normore 2006). Some algal lineages are preferentially found in particular environments, such as the Mediterranean (*Muggia et al.* 2008; Fernández-Mendoza *et al.* 2011), suggesting the presence of habitat-adapted strains.

Recent studies have shown that the adaptive potential of populations is positively associated with the degree of overall genetic diversity. The loss of genetic diversity is therefore a potential threat to the affected population or species (Fitzgerald *et al.* 2011; Pauls *et al.* 2013; Stiebens *et al.* 2013). The ubiquitous presence of cryptic

species (Škaloud & Peksa 2010) suggests that morphospecies-based approaches are likely to underestimate global climate change effects on biodiversity. In particular, widespread species with broad ecological niches are often taxonomically complex entities that represent numerous subspecies or other cryptic diversity units (Leavitt *et al.* 2011). Our understanding of genetic diversity at the level of populations and individuals in lichen associations has remained limited due to the lack of markers with high genetic resolution, e.g. microsatellites. Studying diversity at this scale is relevant because the diversity and stability of algal lineages within a lichen thallus may dictate the adaptive potential of these symbioses to environmental change as has been shown in other organisms (Pauls *et al.* 2013).

So far, algal symbiont diversity in lichens has been assessed at high molecular resolution (microsatellites) in only two species. In the case of Lobaria pulmonaria, the authors found congruent genetic structure between symbionts (Werth & Scheidegger 2012; Widmer *et al.* 2012), with the exception of one study which reported one-to-many associations of symbiont genotypes within thalli (0.3% multiple fungi, 1.6% of multiple algae based on threelocus data sets; Werth *et al.* 2006). In the case of *Parmotrema tinctorum* associated with the green algae *Trebouxia corticola*, *T. higginsiae* and an unknown *Trebouxia* species, the authors found several fungal and algal genotypes in the majority of thalli using four fungus- and five alga-specific microsatellites markers (Mansournia *et al.* 2012). Both studies concluded that multiple photobionts can be transmitted during clonal propagation, in particular due to fusion of propagules from different individuals. However, for *P. tinctorum*, the relatively high frequency of null alleles in the algal PCRs and the algal ITS sequence data indicate that the multiple algal genotypes found within a thallus may represent different *Trebouxia* species, rather than genetic variants of a single algal strain.

Here we apply highly informative microsatellite markers to study intrathalline green algal diversity in lichen associations. Specifically, we assess differences in the intrathalline genetic diversity of symbiotic algae in two unrelated fungal hosts that do not produce vegetative propagules. For this, we selected two species of lichen-forming fungi that are highly selective for *T. decolorans* (Beck & Mayr 2012) and differ in their ecological sensitivity. *Xanthoria parietina* (L.) Th. Fr. is a tolerant, widespread species whereas *Anaptychia ciliaris* (L.) Körb. is sensitive to air pollution and is locally endangered in Europe. Both species reproduce sexually by means of meiotic spores. In lichens, sexual fungal reproduction involves the independent dispersal of the symbionts and de novo formation of the symbiosis. This latter process was shown to

reshuffle the genotypic combinations of partners and to allow for symbiont switching among unrelated taxa (Dal Grande *et al.* 2012; O'Brien *et al*. 2013).

Materials and methods

To investigate intrathalline genetic diversity of the photobiont, three to seven samples from different parts of the same thallus were collected for each specimen, resulting in a total of 96 thallus pieces (51 of *X. parietina* and 45 of *A. ciliaris*) from 20 thalli (10 of each species). We selected only well-defined individuals, without signs of fusion with neighboring thalli. All samples were thoroughly rinsed with tap water to wash off external algae potentially adhering to the thallus. Specimen information (codes, sampling localities and GenBank accession numbers) is given in Table 1.

Host	Sample code	Locality	*N*	GB accession ITS alga	GB accession ITS	Lat	Long
Xanthoria parietina	I1	Lonigo, Italy	5	KJ027684	KJ027704	45.396	11.402
Xanthoria parietina	I2	Lonigo, Italy	5	KJ027683	KJ027703	45.396	11.402
Xanthoria parietina	I3	Lonigo, Italy	5	KJ027682	KJ027702	45.391	11.396
Xanthoria parietina	D1	Frankfurt, Germany	5	KJ027685	KJ027705	50.153	8.765
Xanthoria parietina	D2	Frankfurt, Germany	7	KJ027687	KJ027708	50.153	8.765
Xanthoria parietina	D3	Frankfurt, Germany	5	KJ027686	KJ027706	50.153	8.765
Xanthoria parietina	D4	Frankfurt, Germany	4	KJ027698	KJ027709	50.153	8.769
Xanthoria parietina	D5	Frankfurt, Germany	4	KJ027699	KJ027710	50.155	8.773
Xanthoria parietina	D6	Frankfurt, Germany	7	KJ027688	KJ027707	50.155	8.773
Xanthoria parietina	D7	Frankfurt, Germany	4	KJ027700	KJ027711	50.158	8.773
Anaptychia ciliaris	N1	Rakkestad, Norway	4	KJ027693	KJ027718	59.411	11.368
Anaptychia ciliaris	N2	Rakkestad, Norway	5	KJ027694	KJ027719	59.411	11.368
Anaptychia ciliaris	N3	Rakkestad, Norway	5	KJ027690	KJ027721	59.410	11.370
Anaptychia ciliaris	N4	Rakkestad, Norway	4	KJ027697	KJ027720	59.410	11.368
Anaptychia ciliaris	N5	Rakkestad, Norway	5	KJ027695	KJ027715	59.410	11.369
Anaptychia ciliaris	E1	Campillo de Ranas, Spain	3	KJ027691	KJ027717	41.141	−3.306
Anaptychia ciliaris	E2	Campillo de Ranas, Spain	5	KJ027689	KJ027713	41.141	−3.306
Anaptychia ciliaris	E3	Campillo de Ranas, Spain	5	KJ027696	KJ027714	41.143	−3.304
Anaptychia ciliaris	E4	Campillo de Ranas, Spain	6	KJ027701	KJ027716	41.143	−3.304
Anaptychia ciliaris	E5	Campillo de Ranas, Spain	3	KJ027692	KJ027712	41.141	−3.302

Table 1 Information on the samples used in this study.

N: number of samples per thallus genotyped at 8 microsatellite loci.

Total genomic DNA was isolated using the cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle 1987). Algal and fungal ITS of all 96 samples were sequenced using the universal fungal ITS1F/ITS4 (White *et al.* 1990; Gardes & Bruns 1993) and alga-specific ITS1T/ITS4 (Kroken & Taylor 2000) primers. For PCR protocols see Sadowska-Des *et al.* (2013). Out of the 20 microsatellite loci developed for *T. decolorans* associated with *X. parietina* and *A. ciliaris,* we selected the ten most variable loci (Tde03, Tde04, Tde05, Tde06, Tde07, Tde09, Tde12, Tde14, Tde17, Tde18; Dal Grande *et al*. 2013). We then excluded two markers (Tde04, Tde09) because of inconsistent amplification of DNA extracts from *A. ciliaris*. All samples were therefore genotyped at eight microsatellite loci. All markers used in this study have

been tested on several *Trebouxia strains* (*T. asymmetrica*, *T. corticola*, *T. gigantea*, *T. impressa* and *T. simplex*) and are specific to *T. decolorans* (Dal Grande *et al.* 2013). Fragment lengths were determined on a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA), and electropherograms were analysed with Geneious 5.6 (Biomatters Ltd., Auckland, New Zealand) using LIZ-500 as internal size standard.

Algal ITS phylogenetic analyses For each algal ITS sequence, the 10 highest scoring non-identical BLAST hits were included in the data set. We complemented the alignment with sequences from morphologically identified cultures of *T. decolorans* and other *Trebouxia* species (seven SAG and two UTEX strains). Alignments were generated using MAFFT (Katoh *et al.* 2005) and gaps were removed manually.

Phylogenetic relationships and their confidence values were inferred using maximum likelihood (ML) implemented in RAxML (Stamatakis 2006), and Bayesian inference as implemented in MrBayes 3.1 (Ronquist & Huelsenbeck 2003). All ML searches followed a GTRGAMMA model of molecular evolution. We ran 1000 ML bootstrap pseudo-replicates (BS). For Bayesian analysis, the best-fit model was selected with the corrected Akaike Information Criterion as implemented in jModelTest 2.1.1 (012340+G; Guindon & Gascuel 2003; Posada 2008). All trees were rooted and Bayesian phylograms including posterior probabilities (PP) were computed with four chains and five million generations. One out of every 100 trees was sampled, and the first 12,500 trees were discarded as burn-in. In all analyses, the clade including *T. brindabellae* SAG 2206, *T. angustilobata* SAG2204, *T. suecica* SAG 2207, *T. australis* SAG 2205 and *T. simplex* SAG 101.80 was used as outgroup. ML trees were graphically displayed with FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Microsatellite data analysis Although *T. decolorans* is haploid, multiple alleles were detected at some loci in some samples. Multiple alleles at a particular locus were scored when a second clear peak of the chromatogram was found in the expected size range that was at least one-third the height of the dominant peak (Pettay *et al.* 2011). All microsatellite markers used in this study amplified only a single allele when tested on pure clonal cultures of *T. decolorans* (Dal Grande *et al.* 2013), thus we conclude that multiple alleles per sample correspond to genetically distinct *Trebouxia* individuals within a single lichen thallus. This prevented the construction of multilocus genotypes, resulting in a binary data set of the presence or absence of alleles within samples. We calculated rarefied allelic richness (n = 3) for each locus within each thallus using

ADZE 1.0 (Szpiech *et al.* 2008) and compared the averages over eight loci between hosts.

We investigated partitioning of *T. decolorans* genetic variation between and within hosts, among and within sites (*X. parietina*: Italy, Germany; *A. ciliaris*: Norway, Spain; see Table 1), and among and within populations with hierarchical analysis of molecular retrieved for this study are in bold face. Numbers indicate maximum likelihood bootstrap support values (1000 replicates) above 75%. Thickened branches indicate Bayesian posterior probabilities P0.95 variance (AMOVA), with populations nested within hosts and within sites for each host (9999 permutations) in Arlequin 3.5 (Excoffier *et al.* 2005).

We used hierarchical clustering with the Ward's method on the Euclidean distances from the allele presence/absence matrix to assess relationships among algal individuals. The reliability of clusters was assessed with approximately unbiased P values calculated by multiscale bootstrap resampling (105 replications) using the package pvclust in R (Shimodaira 2004; Suzuki & Shimodaira 2006; R Development Core Team 2011). Clusters with approximately unbiased P value 0.95 were considered strongly supported.

Results

ITS rDNA diversity We found a single fungal and algal ITS haplotype within each thallus. In total, we found seven *X. parietina* and nine *A. ciliaris* fungal ITS haplotypes, and five algal ITS haplotypes. Identical fungal ITS haplotypes were found in four *X. parietina* (D6, D7, I1, I2) and two *A. ciliaris samples* (E1, E2). The most common algal haplotype (indicated with an asterisk in Fig. 1) was associated with four samples of each host species (Fig. 1) and corresponds to the strain M-0102151 (*T. decolorans* ex *Xanthoria parietina* Germany, Maising; GenBank accession JF831923; Beck & Mayr 2012) that was used for microsatellite development.

Phylogenetic analysis of the photobiont The final algal data set included 36 unique sequences. The alignment was 746 bp long and included 201 variable sites. All algal ITS sequences generated in this study grouped with high support (BS = 84%, PP = 0.99) with the sequence of the authentic strain of *T. decolorans* Ahmadjian (UTEX B781). This clade was sister to the morphologically similar species *T. arboricola* SAG 219.1A, as found by Beck and Mayr (2012). Thus we conclude that all photobionts of the studied lichen thalli belong to only one algal species, *T. decolorans*. Photobionts of

X. parietina and *A. ciliaris* are closely related to photobionts associated with other lichen-forming fungi, including several *Xanthoria spp*., *Anaptychia setifera* and *Ramalina fraxinea* (Fig. 1).

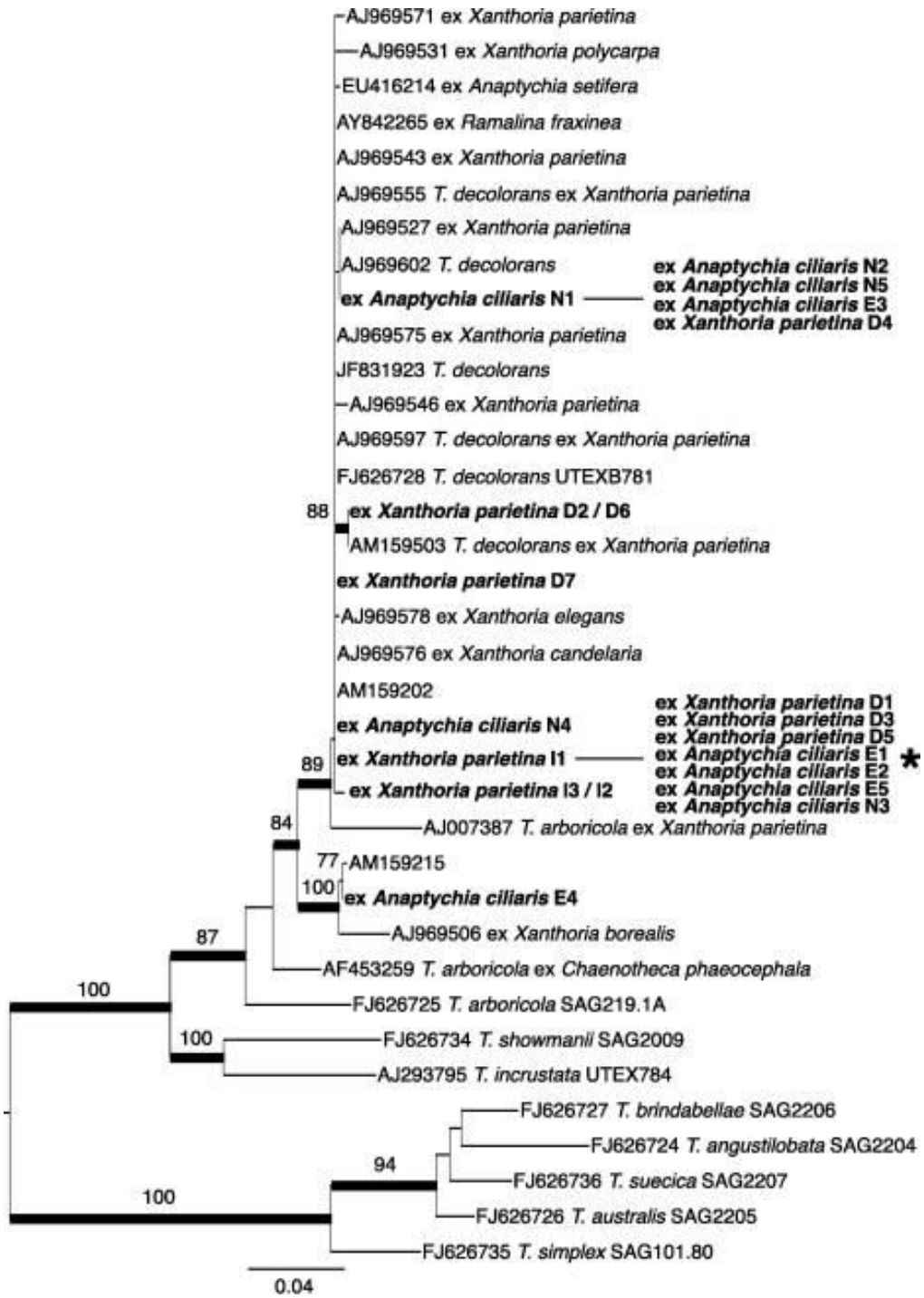

Figure 1 *Trebouxia decolorans* ITS rDNA phylogeny. This is a maximum likelihood tree based on 36 unique sequences. Algal sequences associated with *X. parietina* and *A. ciliaris* retrieved for this study are in bold face. Numbers indicate maximum likelihood bootstrap support values (1000 replicates) above 75%. Thickened branches indicate Bayesian posterior probabilities ⩾0.95.

Microsatellite diversity of the photobiont The majority of algal samples associated with *A. ciliaris* had multiple alleles per locus (11 at one locus, 6 at two loci, 2 at three loci and 2 at four loci), whereas in *X. parietina* only three samples displayed two alleles at one (2 samples) and 5 loci (1 sample). In *A. ciliaris*, three thalli (N2, N3, N4) had monoclonal *Trebouxia* strains. In *X. parietina*, only one thallus (D1) had a monoclonal *Trebouxia* population.

A total of 113 alleles at eight microsallite loci were recovered from *T. decolorans* associated with *X. parietina* and *A. ciliaris*. Of these, 40 alleles were private to *Trebouxia* associated with *A. ciliaris* and 42 to algae in *X. parietina*.

Rarefied allelic richness per locus within thallus ranged from 1 to 1.697 in *A. ciliaris* and from 1.386 to 1.923 in *X. parietina*. A Wilcoxon signed-rank test showed that the two lichen-forming fungi differ in their algal allelic richness (W= 64, p-value = 0.007). In particular, intrathalline photobiont populations in *A. ciliaris* had significantly lower allelic richness than those in *X. parietina* (Fig. 2).

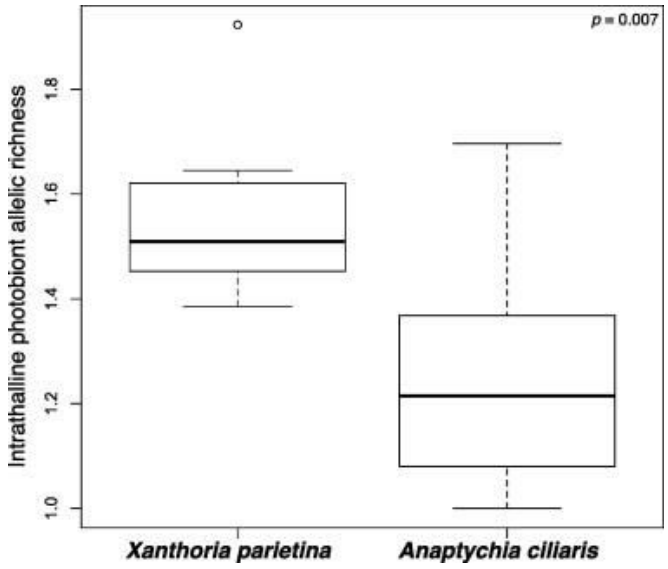

Figure 2 Within-thallus *T. decolorans* allelic richness. Box-plot of intrathalline rarefied allelic richness (N = 3) of *T. decolorans* strains associated with *X. parietina* and *A. ciliaris*. Box-plots depict the median (horizontal line), the 25th and 75th percentiles (bottom and top of the box) and the highest and lowest values (higher and lower whisker).

Differences among thalli accounted for most of the total photobiont allelic genetic variation (76% in the total data set, 55.58% in *X. parietina*, 82.04% in *A. ciliaris*). Differences between fungal hosts accounted for only 6.27% of the total variation. Intra-thalline algal differentiation explained a higher proportion of the algal genetic variance in *X. parietina* (36.44%) than in *A. ciliaris* (17.32%) (Table 2).

Xanthoria parietina sites I1–I3 (Italy)/D1–D7 (Germany) and *A. ciliaris* sites N1–N5 (Norway)/E1–E5 (Spain) were not significantly differentiated suggesting that geographical differentiation in *T. decolorans* is weak.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation indexes	*P*
Between hosts	1	49.768	0.47102	6.27	0.06269	**0.01**
Among thalli within hosts	18	471.801	5.07416	67.53	0.72047	**<0.001**
Within thalli	76	149.619	1.96867	26.20	0.73799	**<0.001**
Xanthoria-host						
Among sites (I1–3, D1–7)	1	34.247	0.55667	7.97	0.07972	0.09
Among thalli within sites	8	177.770	3.88138	55.58	0.63557	**<0.001**
Within thalli	41	104.336	2.54477	36.44	0.60400	**<0.001**
Anaptychia-host						
Among sites (E1–5, N1–5)	1	31.112	0.04756	0.64	0.00637	0.38
Among thalli within sites	8	228.671	6.12907	82.04	0.82681	**<0.001**
Within thalli	35	45.283	1.29381	17.32	0.82570	**<0.001**

Table 2 Analysis of molecular variance of the presence and absence of microsatellite alleles within *Trebouxia decolorans* samples. Genetic variation was partitioned between hosts and among sites within *Xanthoria parietina* and *Anaptychia ciliaris* hosts (*X. parietina* host: I: Italy, D: Germany; *A. ciliaris* host: E: Spain, N: Norway). P values in bold face indicate significance at a = 0.05 following sequential Bonferroni correction.

Hierarchical clustering demonstrated different patterns of photobiont relatedness in the two fungal hosts. Photobionts within each thallus of *A. ciliaris* were closely related (Fig. 3). Contrarily, different photobionts from the same thallus of *X. parietina* did not always group together (Fig. 3). Photobionts from *X. parietina* thalli D3, D4, I1 and a few individuals from thalli D2, D5 and D7 were more closely related to photobionts associated with *A. ciliaris*.

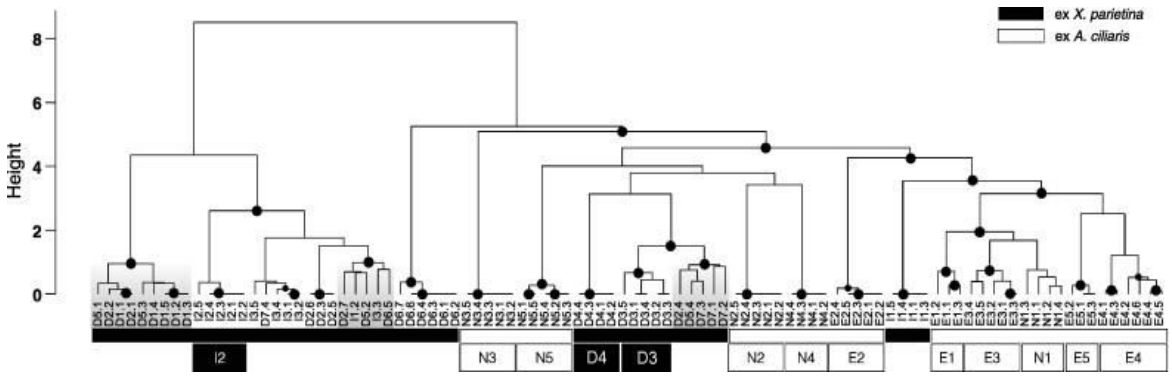

Fig. 3 Cluster dendrogram of T. decolorans individuals. Genetic structure of *T. decolorans* individuals associated with *X. parietina* (black bars) and *A. ciliaris* (white bars) from 20 thalli. Structure was examined considering the presence and absence of all microsatellite alleles within samples. Topology was inferred using hierarchical cluster analysis of Euclidean distances from the allele presence/absence matrix grouped using the Ward's method. Black dots indicate nodes with approximately unbiased probability value ⩾0.95. Black and white boxes with thallus code indicate individuals clustered according to their thallus of origin for *X. parietina* and *A. ciliaris*, respectively. Gray clusters highlight grouping of individuals from different thalli.

The present study contributes to our understanding of intra-species and intra-individual genetic diversity of symbiotic microorganisms. Microsatellite analyses of *T. decolorans* populations revealed the presence of genetically distinct photobionts within thalli of two lichen-forming fungi. While sequence-based markers were monomorphic within each thallus and indicated the presence of only a few algal ITS haplotypes in both analysed species, microsatellites showed that most thalli harbored several photobiont strains. Algae in *X. parietina* mostly had a single multi-locus genotype (i.e., one allele at each locus), differing at each sample site within a thallus, similarly to *Trebouxia* spp. associated with *Parmotrema tinctorum* (Mansournia *et al.* 2012). Conversely, the algal populations in *A. ciliaris* harbored multiple co-occurring alleles at each locus in various parts of the thallus.

Different scenarios could explain the presence of multiple algal strains within a lichen thallus: (i) somatic mutation of rapidly dividing algae; (ii) start of lichenization from a heterogeneous algal pool collected from the environment; (iii) acquisition of genetically different algal strains during the lifetime of the thallus.

The first scenario has been observed in other organisms with complex life histories (see Orive 2001) and it is the most likely in our data set. Our results show that most of the allelic variance is explained by differences among thalli, i.e. photobionts within a thallus are more similar than photobionts from different thalli. This suggests that photobiont diversity is created de novo in both fungal hosts. Somatic mutations were inferred to be the only source of the high genetic diversity of another symbiotic green alga, *Dictyochloropsis reticulata*, although in this case the authors did notfind multiple algal strains within a thallus (Dal Grande *et al.* 2012).

In *X. parietina*, higher allelic richness, higher within thallus differentiation and lower intrathalline relatedness of algal individuals suggest that additional processes influence the genetic diversity of the photobiont. Morphological observations as well as population genetics studies on *T. decolorans* indicate that the second and third scenario may occur in this lichen-forming fungus. Ott (1987) observed that the germinating spores of *X. parietina* form a loose hyphal web that can grow over a large area within a short time and establish contacts with photobionts present on the substratum. *Trebouxia decolorans* is a common and widespread lichen photobiont. There is evidence that *T. decolorans* occurs as free-living organism (Bubrick *et al.* 1984), or as member of epithalline algal communities on widespread crust-forming lichens (Muggia *et al.* 2013), or within faecal pellets of lichenivorous mites (Meier *et al.* 2002). Therefore it is likely that multiple algal strains can be incorporated during de

novo lichen-formation, resulting in less structured, more diverse, intra-thalline algal populations in *X. parietina*.

Environmental uptake of beneficial symbionts without vertical transmission is a strategy well documented in other highly specific symbioses, such as the stinkbug – *Burkholderia*, legume –*Rhizobium*, alder – *Frankia*, arbuscular mycorrhizae and corals –*Symbiodinium* relationships (Yamanaka *et al.* 2003; Kiers & Denison 2008; Andras *et al.* 2011; Kikuchi *et al.* 2011; Pettay *et al.* 2011; Howells *et al.* 2013). In these systems, the lack of compartmentalization allows the maintenance of promiscuous symbioses (Aanen *et al.* 2009). The fact that several *X. parietina* thalli (I1, I3, D2, D5, D6, D7) harbored unrelated algal individuals support the hypothesis of the incorporation of extrathalline photobionts in mature thalli. In support of this hypothesis, morphological studies revealed localized regions of actively dividing fungal and algal cells located along wound margins in thalli of *X. parietina* (Honegger 1996). These anatomical features could facilitate the uptake of external photobionts. Meier *et al.* (2002) also showed that oribatid mites commonly graze upon the upper cortex and algal layer of *X. parietina*, and that their faecal pellets are vectors of viable algal cells.

In conclusion, differences in photobiont populations might be explained by different growth form and ecological preferences of the fungal hosts. Although there are no reports on the regenerative capacity of *A. ciliaris*, it is likely that once an association with a particular photobiont is formed, this symbiont is propagated during marginal thallus growth. The dorsiventral, apically growing lobes may be responsible for the lower diversity and the absence of spatial localization of particular algal genotypes. Our results support intrathalline somatic mutation as the main source of photobiont diversity in *A. ciliaris*. A possible explanation for the presence of multiple co-occurring algal alleles in thalli of *A. ciliaris* is that thalli of this fungus are more long-lived than those of *X. parietina*, allowing more time for mutation to accumulate. This may represent a selective advantage to cope with changing environmental conditions in lichen-forming fungi with low regenerative capacity.

By contrast, *X. parietina* forms wrinkled rosettes of overlapping lobes, with squamulose lobes present towards the center of the thallus resulting from regeneration. Regeneration might enable uptake of new photobiont strains. This more diverse photobiont pool adds to the diversity generated via mutation leading to the observed mosaic of genetically different photobionts within thalli.

The ability to harbor multiple photobiont lineages may contribute to increasing the breadth of the ecological niche of lichenforming fungi (Casano *et al.* 2011). In line

with this concept, we find higher photobiont diversity in *X. parietina*, a Cosmopolitan species that grows on a wide variety of nutrient-rich substrata (Lindblom & Ekman 2006). Fewer photobiont lineages associate with *A. ciliaris*, which is sensitive to SO_2 air pollution and has been decreasing in its range. As the maintenance of high levels of genetic diversity has an adaptive role in several species (Llanes *et al.* 2013), further studies should test whether the use of highly variable loci such as the microsatellites used in this study can help identifying genetic groups with different adaptive responses. Overall a growing body of evidence suggests that symbiont promiscuity is a general feature of lichen associations with *Trebouxia spp.* This has been shown using sequence markers (e.g., Casano *et al.* 2011; Muggia *et al.* 2013), as well as microsatellite markers (Mansournia *et al.* 2012; this study).

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CAPITULO III / CHAPTER III

Differences in sexual aposymbiotic phase of the reproductive cycle in *Parmelina carporrhizans* and *Parmelina quercina*. Possible implications in its reproductive biology

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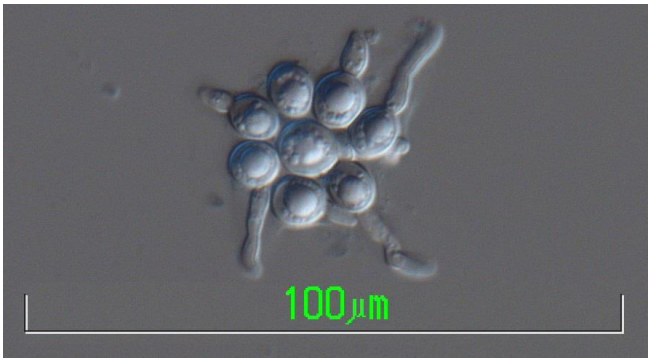

Germinating spores from one *Parmelina carporrhizans* asci at six days after ejection.

ABSTRACT

Our knowledge of ontogenetic development and reproductive strategies in lichen-forming fungi is rather scarce. Here, we aim to advance our understanding of the reproductive biology of *Parmelina carporrhizans* and *P. quercina* for which both mycobiont fungi were cultured in aposymbiotic conditions from ascospores. In *P. carporrhizans* the apothecia needed 48 h to reach a percentage of ejected spores of 98.6%, however, 100% of apothecia of *P. quercina* ejected spores in the first 24 h. In both species, while large apothecia eject more spores than smaller ones and the spore germination percentage was independent of apothecium size. The germination ratio was higher in *P. carporrhizans* (89%) than in *P. quercina* (0.4%). Subsequently, *P. carporrhizans* was grown much more successfully in culture media than *P. quercina*. Therefore, in these specific laboratory conditions, the requirements for ontogenetic development and subsequent development are different between these two species that were recently segregated. We also hypothesize between the relation of spore size-number and dispersion-germination success.

Keywords: axenic culture, aposymbiotic, *Parmelina*, mycobiont, ontogeny, reproductive success

Introduction

Lichens reproduce by vegetative dispersal of both symbionts together (isidia, soralia and thalli fragment) and/or reestablished by ascospores and conidia that disperse aposymbiotically and subsequently encounter compatible photobiont (Sanders & Lücking, 2002). Sanders (2014) observed on nature as macroconidia encircled algae were co-dispersed and germinated early, noticed as this primary photobiont could be replaced by free-living algal cells. Apart from this interesting field approach, our knowledge about lichen-forming fungi reproductive biology is limited and the main difficulties in directly resolving this matter most probably arises from the inability to successfully execute the complete biological cycle under *in vitro* conditions and the lack of traceability of reproductive propagules in nature (Schuster *et al.* 1985; Ott 1987; Sanders 2014). Some molecular approaches showed, with some exceptions, a relative low degree of selectivity between mycobiont and photobiont (see Otálora *et al.* 2010). This versatility in association may have adaptive implications (Engelen *et al*. 2010) and is suggesting a highly dynamic symbiosis (Sanders 2014) where, several lifestyles can allow surviving in different environmental conditions (Gassmann & Ott 2000).

Phylogenetics studies had showed long distant dispersion in several lichen-forming fungi (Werth *et al.* 2007; Werth & Sork 2009; Otálora *et al.* 2010; Amo de Paz *et al.* 2012) and Öckinger *et al.* (2005) demonstrate a relationship between the propagules dispersion capacity and *Lobaria pulmonaria* distribution, a relationship that is modulated by environmental factors. It has been discussed that the sexual and asexual strategies of lichens may represent an ecological trade-off between long distance colonization (small conidia or/and sexual spores) and successful local establishment (vegetative propagules) (Ellis 2012). Martínez *et al.* (2012) reported that this reproductive trade-off pattern depends on environmental changes in precipitation and temperature, and size of the thalli. If we focus on lichen-forming fungi with exclusive sexual reproduction, Tibell (1994) noted that species with small spores have wider global distribution than those with larger spores. But, the complex interaction between wind and other environmental conditions must be considered in dispersion models (Norros *et al.* 2012). Although these and other hypotheses have been established a relationship in lichens between spore size and passive dispersion, as far as we know, few works have been raised between the size and number of spores and the germination success under certain conditions (Norros 2013). However, in plants, “seed size and number theories” have been proposed to explain the advantages of having many small compared with a few large seeds. In general, large seeds have a competitive advantage over smaller seeds as a consequence of their higher

germination rates and greater nutrient reserves (Easton & Kleindorfer 2008a). Consequently, seedlings from large-seeded species should be able to become established under a range of environmental conditions that would not be tolerable for seedlings from small-seeded species. However, smaller seeds may disperse further at the cost of lowered establishment success (Muller-Landau *et al.* 2008). We chose *Parmelina carporrhizans* (Taylor) Poelt & Vêzda and *Parmelina quercina* (Willd.) as a study model for *in vitro* culture, to address the possible differences in the reproductive strategies and sexual behavior (focused in aposymbiotic phase) in two closely related lichen forming fungi with differentiated ecology and distribution. *P. carporrhizans* and *P. quercina* are foliose epiphytic lichen species that reproduces only sexually. No comprehensive studies have been carried out on the distribution of these species since they were considered the same species from 1976 (Hale 1976) until 2007 (*Argüello et al.* 2007a) but personal observations and a body of knowledge give us a good approximation to their distribution (Schauer 1965; Nimis, 1993; Arguello *et al.* 2007; Clerc & Truong 2008; Hawksworth *et al.* 2008; Alors *et al*. 2014). These species, phenotipically similar have a different distribution, although *P. quercina* and *P. carporrhizans* have a distribution mainly coincident with Mediterranean Region. Nevertheless *P. quercina* is able to inhabit as far north as Denmark in Europe, and in dry continental localities within Iberian Peninsula where *P. carporrhizans* is not present. While *P. carporrhizans* grows in more oceanic and humid sites being even present in Great Britain and Macaronesian Islands where *P. quercina* is not present. The morphological differences between these two species are the presence of maculae n the upper cortex of *P. carporrhizans* and wider spores than *P. quercina* which have narrower but longer spores (Argüello *et al.* 2007b). The two species rarely coexist. Recently, *P. carporrhizans* has been confirmed as a hetherothalline with long distant dispersion species using molecular data (Alors *et al*. 2017).

The aposymbiotic culture of lichens and their symbionts using sexual and asexual propagules has improved recently (e.g. McDonald *et al*. 2013; Molina *et al*. 2013). So, Molina *et al.* (2013; 2015) used these biotechnology tools to get valuable direct information about sexual reproductive parameters such as discharge, germination and ontogenetic development of meiospores before associating with algal partner (sexual aposymbiotic phase on reproduction cycle) in *Physconia distorta* (sexual reproduction) and *P. grisea* (mixed species). Honegger & Zippler (2007) deduced the heterothallic nature of several lichens culture on aposymbiotic conditions. Moreover, Molina *et al*. (1997) suggested a co-relationship between the apothecia sexual maturation and size of these, while Denison (2003) mentioned a possible relationship between sexual

maturity and apothecia color. Although there is a considerable number of studies that establish correlation between apothecia production with thallus size (e.g. Hestmark *et al.* 2014) little is known about when those apothecia are sexually mature and if there are observable phenotypic changes in apothecia to indicate the availability to eject germinal spores (Molina *et al*. 1997; Denison 2003). In the current study, we tried to investigate how differs is the ontogeny and to approach to reproductive trade-offs between number/size of spores and reproductive success in these two heterothalline, closely related species with similar but slightly different in morphology, distribution and ecological features. Specifically, we aimed to: 1) compare the ontogenetic development of mycobionts in both species from sporulation to the formation of aposymbiotic aggregates (vegetative mycelia), 2) elucidate if some reproductive parameters (sexual aposymbiotic phase), such as spore ejection and germination could be correlation to apothecia size 3) propose as a working hypothesis about the relationship between number/size of spores and reproductive success (germination and vegetative mycelium development) under in vitro conditions.

Materials and Methods

Lichen material Fresh thallus samples of *P. carporrhizans* growing on *Castanea sativa* (Miller) were collected from Cuevas del Valle (Spain) on 11 October 2012 [40º 18' 28.4" N, 5º 00' 39.0" W] at 1007 m (Maf-Lich 19190, Maf-Lich 19191). Thalli from *P. quercina* were collected from *Quercus ilex* L. from Monfragüe (Spain) on 29 October 2013 [39° 50' 17.47" N, 5° 59' 36.41" W] at 200 m. Samples collected from both species were well developed thalli bearing many apothecia (>20).

Isolation and culture We isolated *P. carporrhizans* and *P. quercina* mycobionts from ascospores discharged following the inverted Petri dish methods of Ahmadjian (1993). The apothecia selected were classified into two size categories: 3-5 mm (small) and >5 mm (large) in diameter. The categories were done based on field observations, considering as large those that are easily visualized. Although, we are transforming a continuous variable into a discrete one, in this way, we facilitate the treatment of the data. We used five large and five small apothecia from each thallus, three thalli per species and 30 apothecia per species. They were mechanically cleaned and washed following the protocols established by Molina and Crespo (2000), the clean ascomata were attached to the inner side of inverted Petri dish lids with petroleum jelly. The plates contained Bold's basal medium (BBM, Deason & Bold 1960) and the ascospores

were discharged upwards onto this inorganic medium, which is suitable for spore germination (Molina *et al.* 1997). The Petri dishes were kept at 18-20ºC in the dark (Molina & Crespo 2000).

After germination, 90 randomly selected uncontaminated multispore agar pieces from *P. carporrhizans* and 30 from *P. quercina* were subcultured on different media: 3% glucose LBM (w/v) (3G-LBM) according to Lilly and Barnett (1951) as modified by Lallemant (1985), 0.2% glucose malt-yeast extract (0.2G-MY) according to Molina *et al.* (2013) and cornmeal agar (CMA) following the manufacturer's instructions (Difco Laboratories, Detroit, MI, USA). Cultures were incubated at 20ºC in the dark (Molina & Crespo 2000). Periodically, mycobionts were examined using a Nikon SMZ800 stereomicroscope (Nikon, Tokyo, Japan) and an Olympus CX40 microscope (Olympus, Japan). For photography, an automatic ring flash system was attached to a Canon 450-D camera lens (Canon, Tokyo, Japan). The observations were carried out using white light and Nomarsky interference contrast. We are assuming that methodological biases are affecting equally both species.

The parameters measured to assess the ontogenetic development of these lichen-forming fungi in culture were: spore morphology, area and volume, germination type, globular growth (diameter after 160 days on different media) and the capacity to produce pigments, which was qualitatively assessed. The sexual parameters studied were: percentage of sexually mature apothecia (percentage of apothecia able to propel spores upward after 24 and 48 h); productivity (number of octet of ascospores ejected by sexually mature apothecia at 48 h); germination (percentage of germinated spore groups), growth capacity after germination on different media (Molina *et al.* 2013, 2015). Growth was analyzed with the program ImageJ (http://imagej.nih.gov/ij/) with images taken under an Olympus SZ30 binocular microscope (Olympus, Tokyo, Japan). We also estimated the relative production (rprod) and relative germination (rger) according to the formula

rprod = (Lprod-Sprod)/(Lprod+Sprod)

rger = (Lger-Sger)/(Lger+Sger)

Where L and S are the values for the large and small apothecia, respectively, ranging between -1 and 1.

Area and volume estimation To estimate the area and volume (size) of spores of both species we took the measures of the length and width of the spores, as described by Argüello *et al.* (2007b), whereby at least 120 spores from six thalli were measured

from each species. The mathematical formula of a spheroid or ellipsoid of revolution was used, which is the geometric figure that most closely resembles the shape of spores.

$S=2\pi a\ (a+\ (b/e)\ \text{arcsine}\ e)$

$V=4/3\ \pi a^2 b$

where *S* is the area and *V* the volume. The spheroid is generated by rotation about the *z* axis of an ellipse with semi-major axis *b* and semi-minor axis *c*, therefore *e* may be identified as the eccentricity.

$e=\sqrt{1-(b^2/a^2)}$

Statistical analysis We developed generalized linear mixed models (GLMMs) to test the hypotheses that spore ejection and germination depended on lichen species (fixed variable with two levels) and thalli (random variable with three levels). GLMMs were fitted assuming a Gaussian distribution error, and for each response variable, we compared three alternative models: (1) null (or intercept only) model ($y = \beta_0$); (2) fixed-effect model (y = species); and (3) a mixed-effect model (y = species + (thallus (species)). The mixed-effect model contained thalli as a random factor nested within species type. In a nested design, each level of the nested predictor was uniquely associated with only one level of the higher-level predictor. With nested data structures, the interaction variance is pooled with the main effect variance of the nested factor (Schielzeth & Nakagawa 2012).

We used an information theory approach (Burnham & Anderson 2002) as an alternative to the traditional hypothesis testing approach (Johnson & Omland 2004) to select the best-fitting models accounting for spore ejection and germination. Models were selected on the basis of Akaike's information criterion corrected for small sample sizes (AICc). The AICc rewards goodness of fit (based on the likelihood function), but also penalizes over-fitting (based on a function of the number of estimated model parameters). For each model set, we calculated the difference in AICc between each candidate model and the model with the lowest AICc (ΔAICc). The best model was that with the smallest AICc, but other models with values of $\Delta AICc < 2$ were considered to receive strong support (Burnham & Anderson 2002; Burnham *et al.* 2011). All analyses were conducted using R (R Development Core Team 2013). We used the lmer function in the lme4 package and the gls function in the nlme package (Pinheiro *et al.* 2016) for fitting models, and the MuMIn package for AICc-based model comparison (Barton 2013).

Results

Maturity of apothecia The spores were ejected in groups of eight (Fig. 1A, and chapter cover page). Almost all the apothecia ejected spores after 48 h, except some small apothecia of *P. carporrhizans* (2.8%). While in the first 24 h only 61.1% of large apothecia and 33.1% of small apothecia of *P. carporrhizans* were sexually mature. The apothecia of all sizes of *P. quercina* ejected in the first 24 h.

Ontogeny *P. carporrhizans* spores had an area of 683.52 ± 1.77 μm^2 and a volume of 1600.25 ± 0.2 μm^3, while *P. quercina* spores had an area of 652.54 ± 5.92 μm^2 and a volume of 1418.69 ± 1.35 μm^3. *P. carporrhizans* spores had a 4% greater volume and 18% greater area than *P. quercina*. The spores began to germinate 3-4 days after sporulation in both species. *P. carporrhizans* showed hyaline meiospores (Fig. 1A), which generated one or two germ tubes growing from the endosporium (Fig. 1B and C). A few days later, short cells with a clearly defined septum were observed (Fig. 1D).

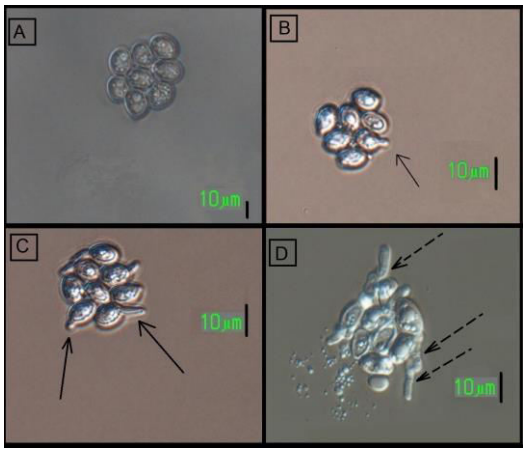

Figure 1 Spore groups of *Parmelina carporrhizans*
A) Aggregate plurisporic 3 days after discharge.
B) and C) Solid arrow indicating the germination tube in spores 4 days after germination.
D) Dashed arrows indicating the septums formed 7 days after germination. Scale bar, 10 µm.

Initially, the development in *P. quercina* was equal to that of *P. carporrhizans*, but after the germ tube was formed, several *P. quercina* hyphae were degenerated (Fig. 2A), many spores remained ungerminated (Fig. 2B), and a substantial number of aborted mycelia were observed after 30 days (Fig. 2C).

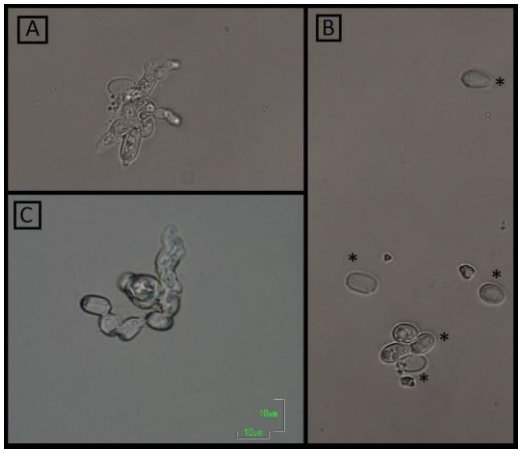

Figure 2
Spore groups of *P. quercina*. A) Germination of spores.
B) Asterisks show the ungerminated spores 10 days after spore ejection.
C) Degenerated culture dying at 30 days. Scale bar, 10 µm.

Spore productivity With respect to the average ejection of plurisporic packs (eight spores from ascus), *P. quercina* had greater productivity than *P. carporrhizans* (637 ± 347 *vs.* 221 ± 360 spores) especially in small apothecia (539 ± 179 *vs.* 95 ± 134 spores) (Fig. 3A). Both species showed positive rprod values (0.17 in *P. quercina* and 0.52 in *P. carporrhizans*), indicating that large apothecia eject more spores than smaller ones. The differences in productivity between large and small apothecia (rprod) were greater for *P. carporrhizans* (Fig. 3B). The mixed-effect model was best at explaining the patterns of spore ejection (rprod, LPA and SPA), in which the species and thallus were both decisive in explaining the process of distribution observed for this fitness parameter (Table 1). The fixed-effect model (including only species) yielded an AICc value lower than that of the null model, but the ΔAICc exceeded 10 units in both cases.

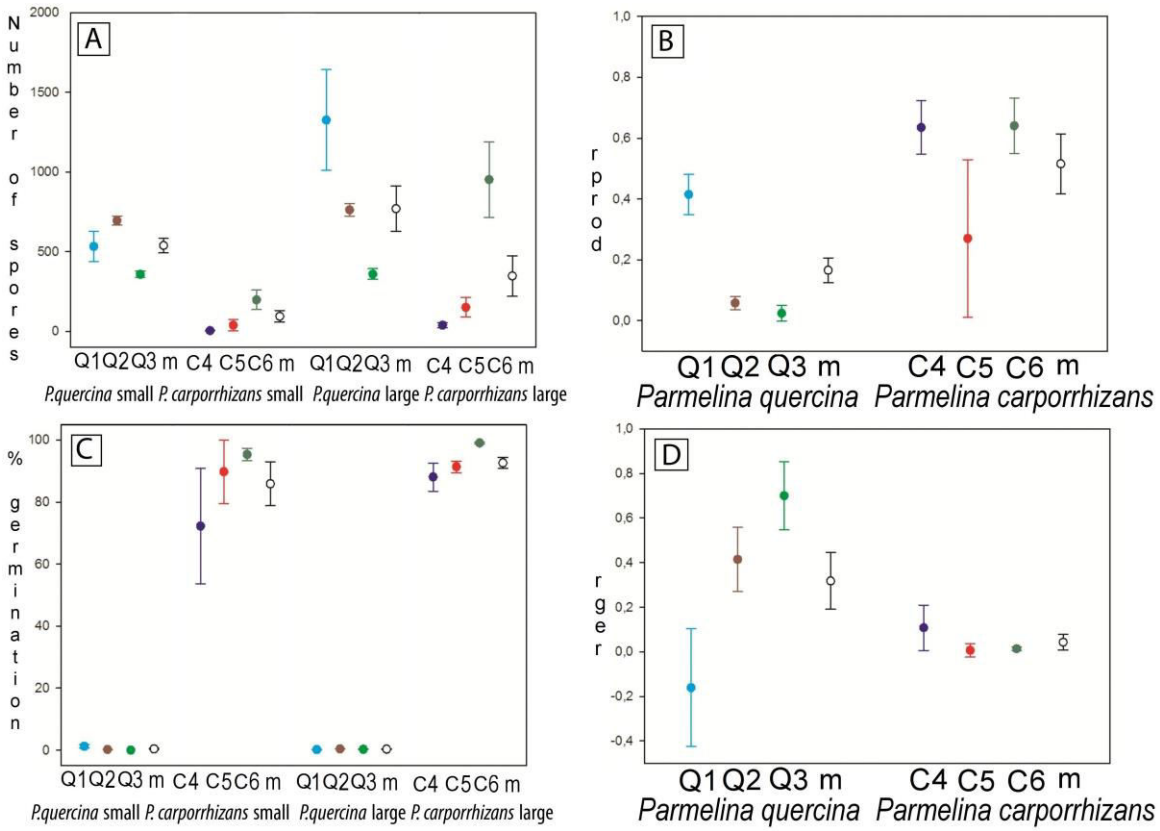

Figure 3 Spore production and germination by thalli and average value (m) of *P. quercina* (Q1, Q2 and Q3) and *P. carporrhizans* (C4, C5 and C6).A) Absolute production of spores. B) Relative production of spores (rprod). C) Percentage germination. D) Relative germination (rger).

Spore germination The percentage of germination was significantly higher in *P. carporrhizans* (89%) than in *P. quercina* (0.4%) for large and small apothecia (Fig. 3 C).

The best model to explain patterns of spore germination (LGA and SGA) was the mixed-effect model (Table 1). The average rger value took average positive values in *P. quercina*, and positive but near-zero values in *P. carporrhizans*. In addition, one of the values of rger for *P. quercina* was negative, showing an uneven individual effect (variability among thalli within species). This value of rger showed that apothecium size did not have a significant and consistent effect on percentage of germination. Moreover, the rger parameter was sensitive to the individual but not the species effect (Table 1, Fig. 3 D). In *P. quercina* the extremely low germination success in absolute value leads to variable and artifact rger values ranging from almost one point of difference in a two points scale (from -1 to 1) as we can see in Fig.3 D.

		rprod		*LPA*		*SPA*		*rger*		*LGA*		*SGA*	
Model	k	AICc	Δ AICc	AICc	Δ AICc	AICc	Δ AICc	AICc	Δ AICc	AICc	Δ AICc	AICc	Δ AICc
Mixed-effect	4	-9	0	335	0	320	0	40	0	320	0	320	0
Fixed-effect	3	7	16	347	12	332	12	54	14	332	12	332	112
Null	2	26	35	361	26	347	27	51	11	347	27	347	27

Table 1 Results of the generalized linear model summarized as Akaike information criterion. The fixed-effect model compares species, and the mixed-effect model compares species and individuals. Respectively, LPA and SPA refer to large and small apothecia production; LGA and SGA represent large and small apothecia germination; Rprod and rger indicate relative production and relative germination

Growth in different culture media The subcultures were transferred to three different culture media in order to ensure growth until aposymbiotic aggregates formation in both species. While growth success was 100% in *P. carporrhizans,* for *P. quercina* was very low (approximately 7%). For this reason, we studied the growth kinetics of *P. carporrhizans* but not of *P. quercina*. *P. carporrhizans* grew faster when subcultures were transferred to 0.2G-MY- and CMA-enriched culture media (averages of about 2.5 and 1.5 μm day^{-1}, respectively), although its development was limited in 3G-LBM (Fig. 4).

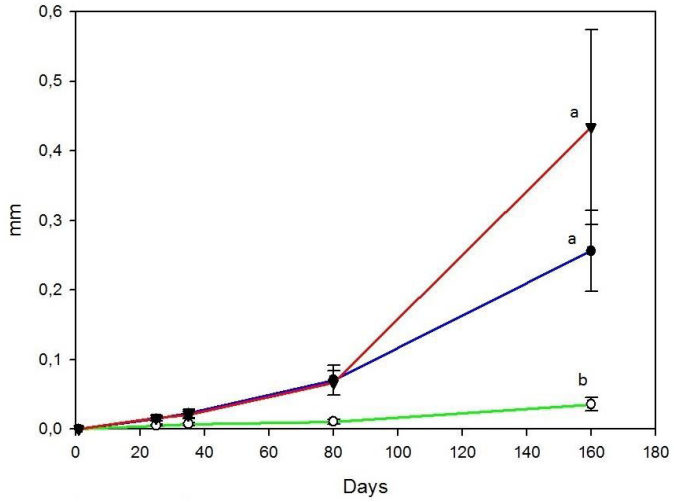

Figure 4
Growth kinetics of *P. carporrhizans* growth (mm) over 160 days. Statistically significant differences are indicated by lowercase letters. Black triangle: 0.2G-MY; black circle: CMA; white circle: 3G-LBM.

The morphology of the *P. carporrhizans* mycelia was similar after 25 days in all three media, but the occurrence of pigmentation was temporarily heterogeneous. While in 3G-LBM the pigmentation appeared after 80 days, in 0.2G-MY appeared on day 25, and in CMA they were fully pigmented by this date (Fig. 5A). The cultures on CMA and 0.2G-MY showed aerial hyphae and dark pigmented hyphae forming aposymbiotic aggregates. After 20-30 days, they also started secreting dark brown liquid that was crystallized on the surface. The cultures formed a hollow callus, which presents a halo of enhanced growth restricted to around the contact zone with culture media. When occasionally germinated spores of quercin grew on culture media, they grew without apparent differences between media (Fig. 5B).

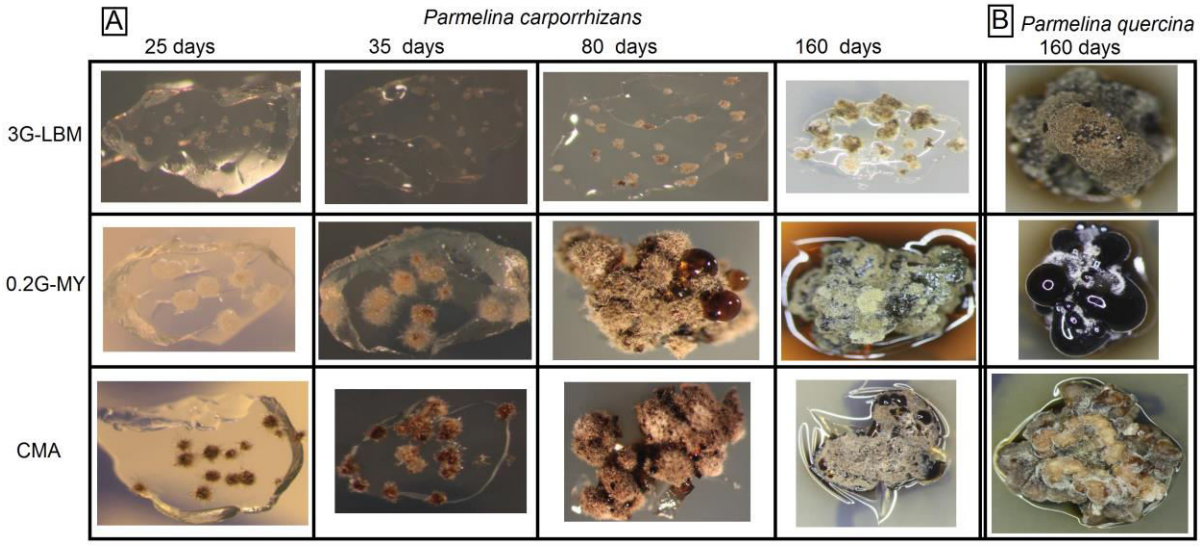

Figure 5 Morphology and pigmentation of cultures.
A) Morphology of cultures of *P. carporrhizans* at four times in three culture media.
B) Morphology of *P. quercina* cultures after 160 days in three culture media.

Discussion

Spore ejection is a phenotypic trait that can vary yield from a few spores to thousands of plurisporic aggregates, depending on several environmental and genetic factors such as species and individual variability, temperature, hydration state, and collection season (Yamamoto *et al*. 1998; Sangvichien *et al*. 2011; Molina *et al*. 2013). Spores of *P. carporrhizans* and *P. quercina* were discharged in a pack or plurisporic aggregates, that is, all the ascospores from the same ascus being ejected simultaneously. This pattern is consistent with those noted in other groups of lichen-forming fungi (Molina & Crespo, 2000; Sangvichien *et al*. 2011; Molina *et al.* 2013). However, the patterns of spore ejection differed between these species since *P. quercina* discharged faster (in the first 24 h) than *P. carporrhizans* which, required an extra day (48 h) to expel spores from almost all the apothecia. The smaller apothecia in *P. carporrhizans* were that caused this delay in spore discharge. *P. carporrhizans* and *P. quercina* ejected between 100 and more than 1000 spores. Together with *Usnea complata* (Sangvichien *et al*. 2011) and *Myelochroa entotheiochroa* (Yamamoto *et al.* 1998), these were the highest spore ejection values recorded so far within the Parmeliaceae family. In terms of absolute discharge in the two species, *P. quercina* ejected more plurisporic packs than *P. carporrizhans*, with significant differences between species. In view of relative productivity (rprod) of both species, rprod was greater than zero, indicating that large apothecia are more productive than smaller ones in both species and therefore the ability to discharge spores seem to depend on the size of the apothecia (Molina *et al.* 1997). The mixed model best explained the variation, implying significant differences between species and individuals. Although the relative productivity differed between species, this parameter also varied within thalli, indicating that caution must be taken when interpreting the observed variance due to the small sampling and the possible of bias as the artificiality of apothecia size classes. The observed variability between thalli within a species is not surprising in fertile lichen populations, as the genetic variability in these populations may be very high, leading to an expectation of high phenotypic variability (Otálora *et al.* 2013). It is worth emphasizing that *P. carporrhizans* and *P. quercina* species are obligate sexually reproducing and heterothallic lichen (Honegger & Zippler 2007: Alors *et al*. 2017). Therefore, although the data seem to indicate that, in fact, larger thalli expel more spores than small ones (Molina *et al.* 1997) to discern the effect of species or individual, more data and samples are needed.

According to the available records on germination time in other Parmeliaceae, *P. carporrhizans* and *P. quercina* germinated seven days earlier than was the case of

Parmelia saxatilis (Molina & Crespo 2000). Further, both species showed bipolar germination similar to *Parmelia saxatilis* (Molina & Crespo 2000) and radial centrifuge growth of plurisporic aggregates (Armaleo 1991). Morphology of the mycobiont mycelia was similar to those described for *Parmelina* species in Honegger & Zippler (2007). The percentage of germination was much higher in *P. carporrhizans* than in *P. quercina*, both in large and small size apothecia. There was no significant difference in relative germination (rger) between species in relation to apothecia size class. In *P. carporrhizans* and *P. quercina* consistent rger values close to zero or negatives showed similar percentage of germination in large and small apothecia the extremely lower germination efficacy of *P. quercina* than *P. carporrhizans*, carries a subsequent growth very low, and thus it was rare for germinated spores ultimately to form vegetative mycelia. In contrast, *P. carporrhizans* grew and developed in different media, CMA and 0.2G-MY being the most suitable for development, with 3G-LBM being less so. It has repeatedly been shown that growth rates for lichen-forming fungi in general depend on culture media (Cordeiro *et al.* 2004; Brunauer *et al.* 2004, 2007; Molina *et al.* 2013; 2015). *P. quercina* expel more asci in less time than *P. carporrhizans*, however, the percentage of germination and the capacity to grow in different media is higher in *P. carporrhizans* than in *P. quercina*. These results show that, under these laboratory conditions, *P. carporrhizans* has greater reproductive success (at least in sexual aposymbiotic phase) than *P. quercina*. This completely different ontogenetic behavior under the same conditions, allows us to assume that the requirements to develop their sexual aposymbiotic phase in nature are different, which might explain why they rarely co-occur. So, two species of the same genus have a completely different ontogenetic development in axenic culture has been previously reported (e.g. Cordeiro *et al.* 2004, Molina *et al*. 2013) and even between genetically very close species as *Diplotomma rivas-martinezii* vs. *D. venustrum* (Molina *et al.* 2002). In these case, the ecology and/or geographical distribution between species was also different. The low sexual aposymbiotic phase success of *P. quercina* under laboratory conditions should not be interpreted as a difficulty to close its biological cycle in the nature, in fact, has a wide distribution and abundance in nature (Clerc & Truong 2008). We only suggest that in nature, the optimal reproductive conditions of *P. quercina* will be different from those of *P. carporrhizans*. These differences provide further evidence that *P. carporrhizans* and *P. quercina* are separate species (Argüello *et al*. 2007a,b; Nuñez-Zapata 2013) as in other closely related species (Molina *et al*. 2002). On the other hand, the area and volume of the spores of these species were quite different, being bigger in *P. carporrhizans* than *P. quercina* (by 4% and 18%, respectively). *P. quercina* ejected faster, spores of low mass (small spore size) and in greater abundance (even small

apothecia ejects hundreds of spores) than *P. carporrhizans*, although the spore germination rate was much lower in *P. quercina* than *P. carporrhizans*. Moreover, *P. quercina* had a low level of success growing in culture media while *P. carporrhizans* produced well-developed vegetative mycelia in two of the three media tested with lesser, but bigger, number of spores. Seem to be than larger spores have a competitive advantage over smaller ones but smaller could move farther than the big ones. If this last premise were true in our study model (Tibell 1994), we can propose a trade-off, since smaller spores travel farther than large ones, (advance), although its rate of germination and its capacity of growth is smaller (disadvance) According to Norros (2013), "the questions whether, how and when to move are not easily separated from the questions whether, how and when to reproduce". So, in wood decay fungi, larger spores had higher initial spore germinability and short distances on the airborne transport. This suggests a trade-off between dispersal distance and establishment probability (Norros 2013). However, Kuparinen *et al.* (2007) report that for small particles, size matters little for their transport by wind. Moreover, other factors as body fruit size, nutritional model and host influence the size of the spore (Kauserud *et al.* 2008). Hottola (2009) suggest that small spore size is related to fragmentation sensitivity in wood decay fungi. Moreover, factors as productivity and germination depend on environmental factors and seasonality (Yamamoto *et al*. 1998; Sangvichien *et al*. 2011). In plants, variation in seed size persists, despite the apparent advantages of being large-seeded (Westoby *et al.* 2002). Easton & Kleindorfer (2008 a,b), using *Frankenia* species as a model, concluded that smaller-seeded species had lower germination success at medium and high temperatures but greater success at low temperatures. Moreover, these species delay germination until they have experienced several days of soil-water contact. That is why models of dispersion and germination that include environmental variables (Norros *et al.* 2012) and field experiences using specific spore traps to measure airborne dispersal and test germination capacity, should be developed. But in the meantime, our results support a working hypothesis in relation to trade off dispersion-germination and size-number spores.

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CAPITULO IV / CHAPTER IV

Characterization of Fungus-Specific Microsatellite Markers in the Lichen-Forming Fungus *Parmelina carporrhizans* (Parmeliaceae)

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Picture from C.G. Boluda

Abstract

• *Premise of the study:* Microsatellite loci were developed to study the lichen-forming fungus *Parmelina* (Parmeliaceae) in different habitats of Western Europe and the Mediterranean for baseline studies to understand the effects of climate change on its distribution.

• *Methods and Results:* We cultured *P. carporrhizans* from ascospores for genomic sequencing with Illumina HiSeq. We successfully developed 11 polymorphic microsatellite markers and associated primer sets and assessed them with 30 individuals from two of the Canary Islands. The average number of alleles per locus was 8.8. Nei's unbiased gene diversity of these loci ranged from 0.53 to 0.91 in the tested populations. Amplifi cation in two closely related species (*P. tiliacea, P. cryptotiliacea)* yielded only limited success.

• *Conclusions:* The new microsatellite markers will allow the study of genetic diversity and population structure in *P. carporrhizans*. We propose eight markers to combine in two multiplex reactions for further studies on a larger set of populations.

Key words: Ascomycota; lichen-forming fungi; microsatellites; multiplex; *Parmelina carporrhizans*; population genetics.

Introduction

Parmelina carporrhizans (Taylor) Poelt & Vêzda (Parmeliaceae) is a sexually reproducing foliose lichen species that has long been considered synonymous with the morphologically similar *P. quercina* (Willd.) Hale. Thus, the geographic distribution and degree of conservation of both species are poorly known (Argüello *et al.* 2007; Clerc & Truong 2008). These two species are largely allopatric but they occasionally overlap, being apparently parapatric depending on the climatic conditions.

Hence they possibly may be used as indicators of climate change. *Parmelina carporrhizans* has an Atlantic-Mediterranean distribution in Europe. It is abundant in the central-western Iberian Peninsula in the humid supra- and mesomediterranean level on deciduous *Quercus* L. vegetation (Argüello *et al.* 2007; Nuñez-Zapata *et al.* 2013). The species also occurs across open forest and in isolated trees above the Canarian Monteverde forest in central Macaronesia from 800 to 1500 m and is locally common on Gran Canaria. Further, *P. carporrhizans* is listed as “vulnerable” on the Red Lists of England and Wales (Church *et al.* 1996; Woods 2010). Despite these conservation concerns, our knowledge of the population genetics of this species is currently limited.

We developed 11 microsatellite markers for high-resolution population studies in *P. carporrhizans* to provide a better understanding of its genetic diversity, gene fl ow, and population structure. The enhanced knowledge will allow us to implement an informed conservation plan and investigate potential impacts of climate change on this narrowly distributed species. In addition, we also investigate whether this set of high-resolution microsatellite markers can be applied to other closely related species in the genus *Parmelina* Hale.

Methods and Results

We isolated the mycobiont of *P. carporrhizans* from ascospores of two thalli (deposited in the herbarium of the Universidad Complutense de Madrid [MAF], Madrid, Spain: MAF-Lich 19191 and MAF-Lich 19192) collected in Cuevas del Valle, Spain (40° 18′ 28.4″ N, 5° 00′ 39.0″ W), in October 2012, following the inverted Petri dish method (Ahmadjian 1993). We germinated spores in Basal Bold Medium (Deason & Bold 1960), and after two weeks these were transferred to corn meal agar (CMA) and malt yeast (Honegger *et al.* 2004), where the cultures were grown for four months.

Prior to DNA extraction, we removed secondary metabolites with acetone, and then crushed the samples with pestles in liquid nitrogen and extracted genomic DNA with the DNeasy Plant Kit (QIAGEN, Charming, California, USA) according to the manufacturer's instructions.

To confirm the identity of the mycobiont cultures, we amplified the internal transcribed spacer (ITS) region of the nuclear rDNA from the axenic cultured tissues. Genomic DNA (10–25 ng) was used for PCR amplifi cations. Primers, PCR, and cycle sequencing conditions were the same as described previously (Argüello *et al.* 2007). Sequencing was conducted on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, California, USA) at Centro de Genómica y Proteómica del Parque Científico de Madrid. The identity of the sequences and specimens were confirmed using the MegaBLAST search function in GenBank. ITS sequences were deposited in GenBank (accession numbers KM357892 and KM357893).

From the extracted DNA, approximately 0.5 µ g of genomic DNA was used to construct an Illumina library using the Nextera XT multiplex paired-end kit (Illumina, San Diego, California, USA). The library was paired-end sequenced using an Illumina HiSeq 2000 with 100 cycles (version 3 chemistry). Standard Illumina protocols (http://www.illumina.com/) were used to generate the library. Sequencing was carried out at the Stab Vida Laboratory (Madan Parque, Caparica, Portugal). Illumina reads were assembled to contigs using the "De novo assembly" option of the CLC Genomics Workbench version 6.0.4 (CLC bio, Aarhus, Denmark). A total of 38,115,484 reads with an average length of 69.06 bases and a total of 2,632,336,717 bases were recovered. De novo assembly produced 31,035 contigs (N50 = 3615 bp) with an average of approximately 73× coverage, which totaled 36.2 Mbp of genome data.

All the contigs were screened for microsatellites using MSATCOMMANDER 1.0.8 (Faircloth, 2008), accepting di-, tri-, tetra-, penta-, and hexanucleotide repeats of

≥ 15. We found 63 contigs containing microsatellite sequences with 15 to 20 repeats (29 dinucleotides, 24 trinucleotides, 7 tetranucleotides, 2 pentanucleotides, and 1 hexanucleotide). From these contigs, we designed short primers of 19–21 bp in length with the program Primer3 using default parameters (Rozen & Skaletsky 2000), expecting some transferability within the genus as reported in other lichen mycobionts (Jones *et al.* 2012; Devkota *et al*. 2014). We excluded contigs with short flanking regions, as well as repeated motifs on the flanking region, and selected primer pairs with amplicons between 100 and 400 bp. Finally, an M13 tag (5′-TGTAAAACGACGGCCAGT-3′) was appended to forward primers for subsequent amplification.

Microsatellite PCRs were performed in a 10- μ L reaction volume containing ~0.5–5 ng of genomic DNA, 1 × Type-it Multiplex Master Mix (Qiagen, Hilden, Germany), 0.15 μ M of reverse primer, 0.01 μ M of M13-tailed forward primer, and 0.15 μ M of dyer–M13-labeled primer (Schuelke 2000). PCRs were carried out with an initial 5-min denaturation at 94 °C; followed by 35 cycles of 94 °C for 30 s, 57 °C for 45 s, and 72 °C for 45 s; and a final extensión of 72 °C for 30 min.

We tested the 24 primer pairs with seven accessions of *P. carporrhizans* from different areas of its distribution range and one accession of *P. tiliacea* (Hoffm.) Hale (MAF-Lich 17252); see Appendix 1 for specific localities. Out of these 24 primers, only 12 pairs successfully amplified all the *P. carporrhizans* samples, and four pairs amplified in *P. tiliacea*. We then tested this subset of 12 primer pairs for variability with 30 samples of *P. carporrhizans* from Gran Canaria and Tenerife (MAF-Lich numbers 19123–19152; Appendix 1), as well as one accession each of *P. tiliacea* and *P. cryptotiliacea* Crespo & Núñez-Zapata (MAF-Lich 19403 and MAF-Lich 19402, respectively). Eight of these primer pairs (Pcar1– Pcar8) amplified all *P. carporrhizans* samples, while the other three (Pcar9– Pcar11) had 3.3–10% missing data. Four of these primer pairs (Pcar3, Pcar5, Pcar7, Pcar9) amplified in *P. tiliacea* and none amplified in *P. cryptotiliacea*. We deposited these 11 primer sequences in GenBank (Table 1); other primer pairs were excluded due to their low amplification rate (<60%). Our limited crossspecies amplifi cation results suggest that it may be possible to use some of these markers in other species of the *P. carporrhizans* clade (Nuñez-Zapata 2013).

Locus	Primer sequences (5′–3′)	Repeat motif	Dye	T_a (°C)	Allele size range (bp)[a]	GenBank accession no.
Pcar1	F: *CATCAAATCATCCGCTACCA R: GGGGAGGTGAGGAGAACAA	$(AC)_{18}$	FAM	57	124–147	KM875582
Pcar2	F: *TCACCATGTGGTAGGGTAGC R: CTGTATCGAACAAGGCATCG	$(GTA)_{15}$	NED	57	206–265	KM875583
Pcar3	F: *TGACCCTGTGACCTCTTGC R: GCCTCGGGTCCATACAGAT	$(AAT)_{17}$	PET	57	109–249	KM875584
Pcar4	F: *AGGAGGGGGTGAAAAAGAGA R: GCTGGTCTTTGCACTCATCA	$(AAGAG)_{16}$	VIC	57	280–318	KM875585
Pcar5	F: *GATGCGTATAGCGGTGCAT R: TTCTGTGGGATGTATTGCAGA	$(AG)_{18}$	FAM	57	227–309	KM875586
Pcar6	F: *GCATTGCATGAGGCTGAAC R: TGCAGTGGCAATCAATGTG	$(CTT)_{15}$	NED	57	203–270	KM875587
Pcar7	F: *CTGGGGTGGTGATTGTGAG R: GCAAGCAGAAAGCAGCAAC	$(AAG)_{19}$	PET	57	120–223	KM875588
Pcar8	F: *GCTTGAATTGGAGGGAAGC R: GAGGCGTGTATGCCTTAACC	$(GAT)_{20}$	VIC	57	372–474	KM875589
Pcar9	F: *GAAACTCCCACCACCGTTC R: AAGCATTTTGGTGCATTGG	$(AG)_{16}$	FAM	57	89–165	KM875590
Pcar10	F: *GCCCTCCAATGAAGGAGTC R: CCTTGGCTGGGATAAGCAT	$(AC)_{16}$	FAM	57	341–390	KM875591
Pcar11	F: *CGATAGCGGAGGATTTTCAG R: GTCTGCGTCGCCTCTAATTC	$(ACTC)_{17}$	FAM	57	250–371	KM875592

TABLE 1 Overview of the microsatellite loci and associated primer sets successfully developed for *Parmelina carporrhizans* and deposited in the National Center for Biotechnology Information (NCBI) database.

Note: T_a = annealing temperature.

a Size range indicates allele size based on two populations collected in the Canary Islands.

* M13 tail: TGTAAAACGACGGCCAGT.

Polymorphism within the eight microsatellite loci that amplified across all *P. carporrhizans* samples was determined by counting the number of alleles and calculating Nei's unbiased haploid diversity (Table 2) using GenAlEx version 6.41 (Peakall and Smouse 2006). The number of alleles ranged from four to 14, and the average unbiased diversity was 0.76, a relatively high number for just 30 individuals from a small geographic area. No identical multilocus genotypes were found among the samples as is expected for a sexually reproducing lichen-forming fungus.

	Total		Gran Canaria ($n = 20$)		Tenerife ($n = 10$)	
Locus	A	H_e	A	H_e	A	H_e
Pcar1	6	0.55	4	0.56	4	0.53
Pcar2	4	0.64	4	0.73	2	0.56
Pcar3	14	0.89	11	0.87	6	0.91
Pcar4	8	0.82	7	0.78	6	0.87
Pcar5	9	0.78	6	0.68	7	0.87
Pcar6	9	0.78	8	0.87	4	0.71
Pcar7	12	0.89	9	0.90	6	0.89
Pcar8	9	0.75	8	0.89	3	0.60
Average	8.88	0.76	7.13	0.79	4.75	0.74

TABLE 2 Number of alleles (A) and Nei's unbiased genetic diversity (He) of the eight polymorphic microsatellite loci that were amplified with 100% success across 30 samples from the Canary Islands.

Conclusions

We developed 11 polymorphic fungus-specific microsatellite markers to facilitate studies of population genetics in *P. carporrhizans.* Eight of the 11 microsatellite primer pairs are being used to analyze *P. carporrhizans* populations. The results from future population genetic studies will help inform us on population responses to global changes, clarify the mechanisms of speciation, as well as defi ne populations of this narrowly distributed species for conservation purposes.

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Voucher (MAF)	Species	Locality	Geographic coordinates	Collectors	Collection dates
16476	*Parmelina carporrhizans*	Canakkale(Tr)	40 ° 06 ′ N 26 ° 55 ′ E	Crespo, Divakar & Candan	15/06/2007
17252	*Parmelina tiliacea*	Tenerife (Es)	28º07'14"N 16º40'19" W	Crespo, Cubas, Santo & Divakar	19/06/2009
19191	*Parmelina carporrhizans*	Avila (Es)	40° 18′ 28″ N 05° 00′ 39″ W	Crespo, D. Alors & C. Ruibal	11/10/2012
19404	*Parmelina carporrhizans*	Tenerife (Es)	28º27'11"N 16º24'55"W	Crespo, Cubas, Santo & Divakar	22/06/2009
19405	*Parmelina carporrhizans*	Gran Canaria (Es)	28º01'50"N 15º37'12"W	Crespo, Cubas, Santo & Divakar	19/06/2009
19406	*Parmelina carporrhizans*	Gran Canaria (Es)	28º00'01"N 15º32'29"W	Crespo, Cubas, Santo & Divakar	17/06/2009
19407	*Parmelina carporrhizans*	Tetouan (Ma)	35º20'43"N 05º22'20"W	Alors & Boluda	22/10/2013
19408	*Parmelina carporrhizans*	Gran Canaria (Es)	28º01'29"N 15º35'15"W	Crespo, Cubas, Santo & Divakar	18/06/2009
19402	*Parmelina cryptotiliacea*	Agadir (Ma)	30º38'51"N 09º40'34"W	Alors & Boluda	23/10/2013
19403	*Parmelina tiliacea*	Azilal (Ma)	33º25'40"N 05º11'26"W	Alors & Boluda	20/10/2013
19123-19143	*Parmelina carporrhizans*	Gran Canaria (Es)	27º59'21"N 15º35'33"W	Crespo, Cubas, Santo & Divakar	22/06/2009
19144-19152	*Parmelina carporrhizans*	Tenerife (Es)	28º27'11"N 16º24'55"W	Crespo, Cubas, Santo & Divakar	23/06/2009

Appendix 1 Voucher information for specimens of *Parmelina carporrhizans*, *P. cryptotiliacea*, and *P. tiliacea* used in this study. All the specimens are deposited in the Lichen section of MAF herbarium, Faculty of Pharmacy, Complutense University, Madrid Spain (MAF-Lich). The first eight samples were tested against all 24 microsatellite primer pairs. The last 32 samples were tested against a subset of 12 microsatellite primer pairs (see Methods and Results). Abbreviations: Tr = Turkey, Es = Spain; Ma = Morocco

CAPITULO V / CHAPTER V

Panmixia and dispersal from the Mediterranean basin to Macaronesian islands of a macrolichen species

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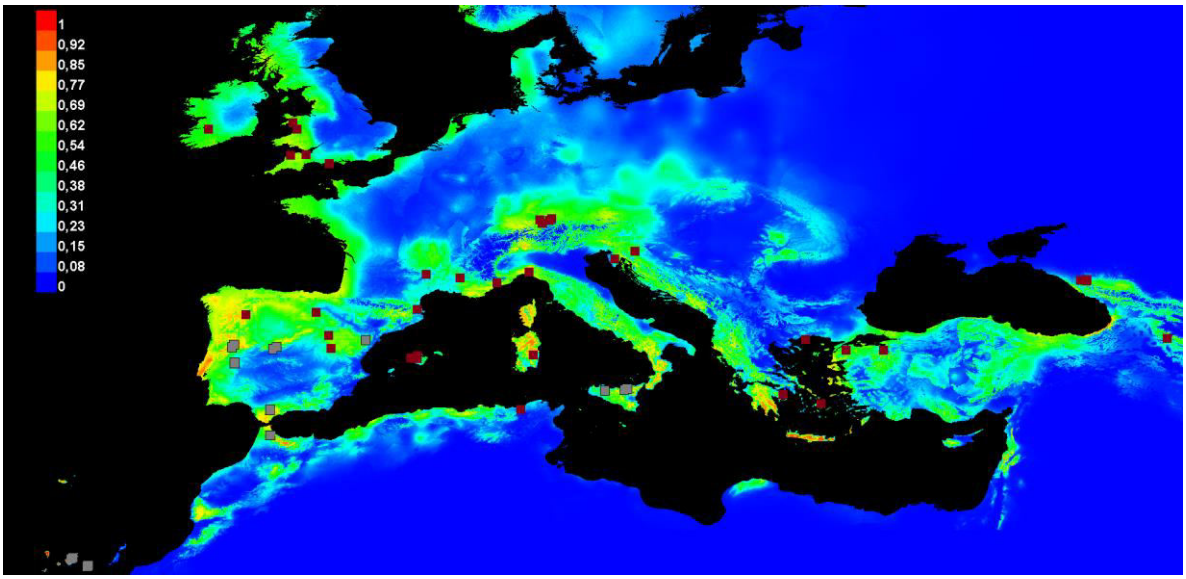

Map of predicted potential distribution of *Parmelina carporrhizans* performed in MaxEnt. Input GPS data are doted in grey (this study) and garnet (MAF-Lich and gbif database) climatic variables were obtained from WorldClim at www.worldclim.org.

Abstract

The Mediterranean region, comprising the Mediterranean Basin and the Macaronesian Islands, represents a center of diversification for many organisms. The genetic structure and connectivity of mainland and island microbial populations has been poorly explored, in particular in the case of symbiotic fungi. Here we investigated genetic diversity and spatial structure of the obligate outcrossing lichen-forming fungus *Parmelina carporrhizans* in the Mediterranean region. Using eight microsatellite and mating-type markers we showed that fungal populations are highly diverse but lack spatial structure. This is likely due to high connectivity and long distance dispersal of fungal spores. Consistent with low levels of linkage disequilibrium and lack of clonality, we detected both mating-type idiomorphs in all populations. Furthermore we showed that the Macaronesian islands are the result of colonization from the Mediterranean basin. The unidirectional gene flow, though, seemed not to be sufficient to counterbalance the effects of drift, resulting in comparatively allelic poor peripheral populations. Our study is the first to shed light on the high connectivity and lack of population structure in natural populations of a strictly sexual lichen fungus. Our data further support the view of the Macaronesian islands as the end of the colonization road for this symbiotic ascomycete.

Keywords: *Parmelina carporrhizans*, microsatellites, gene-flow, founder event, Mat genes

Introduction

Cryptic biodiversity is an essential component of biodiversity that will be considerably affected by global climate change in the next decades (Bálint *et al.* 2011). In recent years molecular genetics has shown that cryptic species are more common across organismal groups than previously thought (Jörger & Schrödl 2013). While there is an increased awareness of the existence and importance of cryptic species, there is a general lack of studies on the amount and distribution of genetic diversity within and among populations of these lineages (Dincă *et al.* 2013). The future of a species, in terms of evolutionary potential, depends on its genetic diversity. This can be affected by the spatial distribution of the species as it influences the amount of gene flow among populations (Frankham *et al.* 2002). Understanding how genetic structure is spatially distributed, within and between populations, is important because it reflects the biology and history of species and determines their evolutionary potential (Excofier *et al.* 2007; Pfenninger *et al.* 2012).

Lichen-forming fungi are a successful group of nutritionally specialized fungi that form obligate symbiosis with photosynthetic partners. Molecular studies of lichen-forming fungi have repeatedly indicated that numerous distinct lineages, i.e. cryptic species, can be hidden under a single taxon. This shows that species diversity of lichen forming fungi might be vastly underestimated, mainly because of the plasticity of morphological and chemical characters and the frequency of homoplasy and convergence of characters (Crespo *et al.* 2009; Lücking *et al.* 2014; Leavitt *et al.* 2015). In the past decades, the use of DNA-based molecular phylogenies for unraveling the hidden diversity in lichen-forming fungi has become increasingly common. However, investigations of these cryptic lineages at the population level are still far less common (Crespo & Lumbsch 2010).

Lichen-forming fungi are interesting subjects for population genetics studies. One of the reasons for this is the vast range of reproductive strategies, i.e., from purely asexual to exclusively sexual, that could differentially shape their population structures (Werth 2010), and peculiar distributions, i.e. across contrasting environmental conditions, which are expected to experience selective pressures. Further, sexual species can either be homothallic (self-fertile), i.e. carrying both required compatible mating types within the same individual, or heterothallic (outcrossing), i.e. requiring the fusion of two individuals with different mating types (Turgeon & Yoder 2000). The expectation is that among sexual species, heterothallic species will harbor more genetic variability than self-fertile ones as each sexual reproductive event may generate new genotypes. Furthermore, exclusively

sexual species should, in principle, exhibit less genetic structure than purely asexual ones because sexual spores are assumed to disperse over larger distances than asexual propagules, thus homogenizing genetic structures (Werth 2010; Singh *et al.* 2015). Overall, the effect will eventually depend on the relative frequency of sexuality over clonality, thus indirectly on the relative abundance of compatible mating types in populations for outcrossing species, and on the spatial dispersal range of the reproductive propagules (Singh *et al.* 2015; Murtagh *et al.* 2000; Dyer *et al.* 2005).

For investigating intra-population variability and inferring the role of reproductive strategies in shaping population dynamics, highly variable molecular markers such as microsatellites (SSRs) are one of the most appropriate tools, especially for highly clonal organisms such as lichens (Arnaud-Haond *et al.* 2006). In particular, SSRs are suitable markers for studying hierarchically structured genetic diversity as their high level of polymorphism ensures a high yield of genetic variability (Werth 2010). However, only a few studies are available which have analyzed the population genetic structure of lichen-forming fungi with these markers. In the few species for which they have been utilized they have revealed high levels of intra-population polymorphism where traditional “variable” nuclear ribosomal markers (e.g., ITS and other ribosomal loci) had revealed little (Widmer *et al.* 2012; Zoller *et al.* 1999). In the case of the exclusively sexual species *Buellia frigida*, a crustose lichen of continental Antarctica, an SSR-based study revealed high intra-population diversity, local population differentiation and limited dispersal, probably resulting from harsh habitat conditions and scarce spatial and temporal habitat availability (Jones *et al.* 2015). Conversely, in the case of the predominantly asexual lichen-forming species *Lobaria pulmonaria*, SSR-based studies have shown highly clonal and structured populations, resulting from low level of recombination and limited dispersal capacity of vegetative propagules (Werth *et al.* 2006; Dal Grande *et al.* 2012). Higher population structure seems to be a common trend of vegetatively reproducing lichens as shown in several studies on other systems (e.g., *Parmotrema tinctorum,* Mansournia *et al.* 2012; *Parmelina tiliacea,* Nuñez-Zapata *et al.* 2015). *Lobaria pulmonaria* is also the only lichen species for which population-level data on the distribution of mating-type idiomorphs is available so far (Singh *et al.* 2015). This study showed a link between significantly unbalanced MAT-gene distribution and high levels of clonality in populations, i.e. lower intra-population genetic diversity. However, given the paucity of SSR-based studies on lichen-forming fungi with different reproductive strategies and the lack of studies on the

distribution of mating types in natural populations, it is not possible to draw general trends for the effects of reproductive modes on lichen population structure.

In this study, we analyzed intra-species genetic diversity, population structure, and mating-type distribution in natural populations of the obligate outcrossing lichen forming fungal species *Parmelina carporrhizans* (Honegger *et al.* 2007). *Parmelina carporrhizans* is a foliose, epiphytic macrolichen with the center of distribution in the Mediterranean region. The Mediterranean region, comprising the Mediterran basin and the Macaronesian islands, is an important centre of diversification for many plant and animal species (Patarnello *et al.* 2007; Warren *et al.* 2015; Mairal *et al.* 2015). However, in the case of lichen-forming fungi, the population structure and connectivity of mainland and island populations in this region have been largely unexplored. We thus aimed at exploring the predictions of two key hypotheses: i) that the sexual reproductive mode of the species leads to high intra-population genetic diversity, high mainland island connectivity and low population structure, and ii) that high frequency of sexual reproduction is reflected in a balanced representation of mating-type idiomorphs in the populations.

To test these hypotheses, we used highly variable, species specific microsatellite and mating-type specific markers. First, we analyzed the species population genetic diversity and evaluated how it is hierarchically and spatially partitioned. We assessed the extent to which alleles are geographically restricted or shared across regions. Second, we calculated the relative abundance of the two mating-type idiomorphs in the populations. Self-incompatible species require the presence of both mating types in the population for sexual reproduction. Third, we estimated migration rates among mainland and island groups. Combining genetic diversity and gene flow estimates we inferred whether the distribution of *P. carporrhizans* is a result of a migration from the Macaronesian Islands to the continent or from the continent to Macaronesian Islands.

Material and methods

Sampling collection We sampled *P. carporrhizans* from 11 localities to extensively cover the south-western distribution range of the species, from the Macaronesian islands and Northern Africa, to Southwestern Europe where the species is most abundant (Fig. 1). From each population we collected 8-29 samples (mean 20) for a total of 220 individuals (Table 1). In smaller populations, we collected samples from all colonized trees within the forest stand. In larger populations, we sampled up to ten nearest neighbor trees from the geographic center of a population. From each tree, we collected up to three thalli from

different branches or of the trunk. Samples were air dried until further treatment. A list of the samples included in this study is given in Supplementary Table S1, together with voucher information.

Figure 1 Map of sampling locations of *Parmelina carporrhizans*. The map was generated using DIVA-GIS v7.5.

DNA extraction and PCR conditions Small pieces (0.5 cm^2) of terminal lobes of freshly collected thalli or recent herbarium specimens were used for DNA isolation with the DNeasy Plant Minikit (Qiagen, Hilden, Germany).

We genotyped each specimen at eight fungus-specific microsatellite markers (Pcar1-8). Details of the markers, PCR conditions and multiplexing are given in ref. (Alors *et al.* 2014). Fragment lengths were determined on a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Electropherograms were analyzed with Geneious7.1.9 (Kearse *et al.* 2012) using LIZ-500 as internal size standard. Missing data were ignored in all subsequent analyses except the analysis of population genetic structure (see below, DAPC). For the analysis of population genetic structure, missing data were replaced with the mean frequency of the corresponding allele computed on the whole set of individuals to avoid adding artefactual between-group differentiation (Jombart *et al.* 2010).

MAT genes: primers design and amplification For designing *P. carporrhizans*-specific MAT-genes primers we obtained partial MAT1-1 and MAT1-2 sequences from the scaffolds of the *P. carporrhizans* genome (Alors *et al.* 2014) via blastx using as query mating-type sequences of *Xanthoria elegans*, *Xanthoria polycarpa* (Scherrer *et al.* 2005),

and *Lobaria pulmonaria* (Singh *et al.* 2012). Primers were designed with Primer 3 Plus (Untergasser *et al.* 2007). The newly designed MAT1-1 primers (Alpha-F: GATCAGCCTCGTTCAACCAT, Alpha-R: TAGTGTGCAGGCTCGATGAC) amplified a 389-bp fragment around the alpha-box of the MAT1-1 gene. The MAT1-2 primers (HMG-F: AAGAAGACAAGGTCGCTCGT, HMG-R:CTTGCGAGGCTGGTACTGAT) amplified a 282-bp fragment around the HMG-box of the MAT1-2 gene.

To identify the mating-type idiomorph of each sample, we performed multiplexed PCR reactions in a total volume of 25 µL with 0.65 µL of each MAT1-1 primer (10µM), 0.6 µL of each MAT1-2 primer (10µM), 0.4 µL dNTP's (10mM) and 0.5 µL (1U/µL) Taq polymerase and 2.5 µL of 10-50 ng DNA. PCR reactions were performed with an initial denaturalization at 94 ºC for 4 min, followed by 10 cycles of denaturalization step at 95ºC for 10 sec, annealing at 55 to 50 ºC for 20 sec and extension at 72ºC for 30 sec, followed by 30 additional cycles with annealing temperature of 50 ºC for 20 sec and an additional extension at 72ºC for 5 min. We tested if both idiomorphs were evenly distributed in the entire area and in each individual sampling locality with X^2 tests.

Genetic diversity Within-population genetic diversity was calculated by estimating allelic richness and private allelic richness using a rarefaction approach, implemented in ADZE (Szpiech *et al.* 2008). We also calculated Nei's unbiased haploid diversity (uh) using GenAlEx version 6.41 (Peakall & Smouse 2006).

To analyze the level of linkage disequilibrium we estimated rBarD (unbiased index of association Agapow et al. 2001) within populations and among loci with the function *poppr* of the R package *poppr* v.2.1.1 (Kamvar *et al.* 2014; Kamvar *et al.* 2015). This index detects signatures of multilocus linkage, indicating association between alleles at different loci and clonal reproduction within populations. Significant departure from the null model of no linkage among markers was tested using 999 permutations at a significance level of 0.05.

To quantify genetic differentiation among and within populations we carried out a global analysis of molecular variance (AMOVA) as a weighted average over loci using Arlequin v3.5 (Excoffier & Lischer 2010). Significance was obtained via a non-parametric permutation procedure (20,000 permutations). Additionally, pairwise genetic differences between populations (FST) were calculated using Arlequin v3.5.

Population genetic structure To detect population structure we used the multivariate, model-free method DAPC (Discriminant Analysis of Principal Components Jombart *et al.*

2010). We chose to use this method because, contrary to a STRUCTURE-like approach (Pritchard *et al.* 2000), it is not based on the assumption of unlinked markers and panmictic populations, which are highly unlikely in clonal or partially clonal organism, such as lichens. Clustering on individuals was performed using the R package *adegene*t 2.0.1 (Jombart 2008; Jombart *et al.* 2010; Jombart & Ahmed 2011)

Spatial analysis We tested for correlation between genetic and geographic distances by performing an isolation by distance (IBD) analysis with the package *adegenet* in R. We used a Mantel test to check for correlation between Edwards' distances and Euclidean geographic distances among populations with the *mantel.randtest* function. To test whether the correlation between genetic and geographic distances is a result of a continuous or patchy cline of genetic differentiation we plotted local densities of distances to disentangle between the two processes. Local point density was measured using a 2-dimensional kernel density estimation with the function *kde2d* and plotted using a customized color palette in the R package MASS (Venables & Ripley 2002). In contrast to a mantel test, which assesses overall correlation of genetic versus geographic distance, the density kernel function looks for underlying genetic structure that may confound or help explain the observed correlation between the two distances.

To further analyze the overall spatial pattern of alleles and test for areas with geographically restricted alleles (GRAs, sensu Widmer *et al.* 2012), we performed spatial analyses of shared alleles (SAShA, Kelly *et al.* 2010). This method compares the spatial arrangement of allelic cooccurrences with the expect pattern under panmixia given the same spatial sampling, and it is particularly appropriate for species with high gene flow and subtle genetic differentiation, such as microbes. GRAs were identified following Widmer *et al.* (2012). Briefly, we calculated central geographic x- and y-coordinates (centroids) and the corresponding standard deviations for each allele at each locus. We then used a bootstrap approach randomly subsampling N x and y coordinates (N = no. of individuals carrying a given allele) to infer null distribution and 95% confidence intervals (CI) of each centroid position. Alleles with centroid falling outside the 95% CI were considered as significantly geographically restricted.

For each data set (overall data set, GRAs) we calculated the observed distribution of geographic distances between allelic occurrences in the overall data set and per marker and compared it with the panmictic distribution. To test for significant deviations of the

observed mean distances from the expected under panmixia, we performed 1,000 nonparametric permutations of the allele-by-location data sets.

Analysis of gene flow To test for the presence and infer the directionality of gene flow between the Macaronesian islands and the mainland populations, we used the coalescent-based method MIGRATE-N 3.2.6 (Beerli & Palczewski 2010). We estimated mutation scaled immigration rate (M), assuming identical but unknown mutation rates (μ) in all populations. Bayesian estimates of M and θ were obtained under an unconstrained migration model with variable θ for each pair of populations separately. We used a uniform prior on both θ (0.0-0.50) and M (0.0-50). A Metropolis-coupled Monte-Carlo chain with static heating was run for 50×10^6 generations, recording every 100 Kth step after a burn-in period of 5×10^4 generations. Convergence was monitored with Tracer (http://beast.bio.ed.ac.uk). All effective sample sizes of the MCMC chain were larger than 10^4.

Results

Mating-type idiomorph distribution We successfully amplified the MAT-genes fragments in 93% of the samples. In each sample we found a single band corresponding to one idiomorph (Fig. S1, Supplementary Material). These results are consistent with a heterothallic organization of MAT genes in *P. carporrhizans*.

The overall difference in frequency of the mating-type idiomorphs was not significantly different, with MAT1-1 found in 55% of the sample, and MAT1-2 in 45% of the samples. Only two populations (Morocco and Herbés) had a significantly skewed higher frequency of MAT1-1 idiomorphs (Table 1).

Population	N	MAT1-1: MAT1-2 (%)	χ^2	uh mean (st. error)	rBarD	AR mean (st error)	PAR mean (st. error)	Regional AR mean (st. error)	Regional PAR mean (st. error)
Tenerife	25	44:56	0.7	0.744 (0.060)	−0.002	4.256 (0.388)	0.447 (0.122)	11.486 (1.787)	3.095 (0.742)
Gran Canaria 1	20	55:45	1	0.785 (0.042)	**0.045**	4.596 (0.363)	0.531 (0.230)		
Gran Canaria 2	20	59:41	2	0.818 (0.040)	−0.001	4.798 (0.444)	0.489 (0.137)		
Morocco	20	65:35	**4.5**	0.848 (0.054)	−0.004	5.521 (0.572)	0.872 (0.270)	19.067 (2.529)	10.676 (1.465)
Cádiz	8	50:50	0	0.826 (0.030)	**0.288**	3.973 (0.247)	0.551 (0.191)		
Marvão	21	55:45	1	0.859 (0.035)	−0.005	5.524 (0.454)	0.706 (0.171)		
Covilhã	17	59:41	2	0.872 (0.027)	0.000	5.623 (0.310)	0.875 (0.243)		
Famalicão	29	50:50	0	0.874 (0.032)	**0.043**	5.695 (0.361)	0.844 (0.138)		
Gredos	25	47:53	0	0.893 (0.033)	0.005	6.047 (0.433)	1.072 (0.173)		
Herbés	14	73:27	**10.33**	0.886 (0.035)	**0.097**	5.786 (0.524)	0.856 (0.175)		
Sicily	21	55:45	0	0.898 (0.018)	0.008	5.860 (0.280)	0.878 (0.136)		

Table 1 Population genetic parameters for eight microsatellite markers and MAT genes in 11 populations of *P. carporrhizans*. N: number of samples; MAT1-1:MAT1-2: mating-type idiomorph ratio within population; χ 2: chi square test of MAT proportions; uh: unbiased haploid genetic diversity; rBarD: unbiased measure of linkage disequilibrium; AR: rarefied allelic richness (N = 8 samples per population, N = 63 per region); PAR = rarefied private allelic richness (N = 8 samples per population, N = 63 per region). Values in bold represent significant P values (α = 0.05).

Genotypic diversity All eight microsatellite loci were highly polymorphic. The analysis of 220 samples with eight microsatellite markers resulted in a total of 220 microsatellite alleles, ranging from 16 alleles (Pcar1) to 46 alleles (Pcar7) (Table S1). No identical multilocus genotypes (MLGs) were found between individuals. The mean frequency of missing data per locus was 3.0% with a range of 0.4% (Pcar2) to 10% (Pcar8) (Table S1).

Overall we found a high unbiased haploid diversity (mean uh = 0.846), ranging from 0.744 (Tenerife) to 0.898 (Sicily). Populations had high population allelic richness (mean = 5.244), ranging from 3.973 (Cádiz) to 6.047 (Gredos). Private allelic richness was moderately low in all populations, ranging from 0.447 (Tenerife) to 1.072 (Gredos). At the regional level, the Macaronesian Islands had lower allelic (11.486) and private (3.095) allelic richness than the mainland populations (19.067 and 10.676, respectively) (Table 1).

We detected low levels of linkage disequilibrium (LD) across populations (mean = 0.069). Low but significant LD values were found in four populations, namely Gran Canaria 1 (rBarD = 0.045, P = 0.014), Cádiz (rBarD = 0.288, P = 0.001), Famalicão (rBarD = 0.043, P = 0.001) and Herbés (rBarD = 0.097, P = 0.002) (Table 1). The AMOVA analysis indicated that most of the molecular variation in P. carporrhizans populations occurs within populations (92.1%), with lesser amounts among populations within regions (2.3%) and among regions (5.6%) (Table 2). Permutation tests (based on 20,000 permutations)

suggest that differences among populations (FST = 0.079) and regions (FCT = 0.056) are not significant (P = 0.11 and 0.08, respectively).

Source of variation	df	Sum of squares	Variance components	% variation
Among regions	1	23.39	0.20	5.57
Among population within regions	9	44.88	0.08	2.29
Within populations	209	683.13	3.38	92.14
Total	219	751.40	3.67	

Table 2 Global Analysis of Molecular Variance (AMOVA) as a weighted average over 8 loci carried out to

As expected, pairwise FST values between populations were low, ranging from 0 to 0.023 (Table 3) and only two pairwise comparisons (Cádiz vs Famalicão and Gredos, both FST = 0.016) were statistically significant.

Population	Tenerife	Gran Canaria 1	Gran Canaria 2	Morocco	Cádiz	Marvão	Covilhã	Famalicão	Gredos	Herbés	Sicily
Tenerife	—	—	—	—	—	—	—	—	—	—	—
Gran Canaria 1	0.002	—	—	—	—	—	—	—	—	—	—
Gran Canaria 2	0.004	0.003	—	—	—	—	—	—	—	—	—
Morocco	0.004	0.003	0.005	—	—	—	—	—	—	—	—
Cádiz	0.018	0.016	0.013	0.019	—	—	.	—	—	—	—
Marvão	0.002	0.000	0.003	0.003	0.016	—	—	—	—	—	—
Covilhã	0.002	0.000	0.003	0.003	0.017	0.000	—	—	—	—	—
Famalicão	0.002	0.000	0.003	0.003	**0.016**	0.000	0.000	—	—	—	—
Gredos	0.002	0.000	0.003	0.003	**0.016**	0.000	0.000	0.000	—	—	—
Herbés	0.007	0.005	0.008	0.008	0.023	0.005	0.005	0.005	0.005	—	—
Sicily	0.002	0.000	0.003	0.003	0.016	0.000	0.000	0.000	0.000	0.005	—

Table 3 Pairwise FST values for 11 populations of *P. carporrhizans*. Significant values (20,000 bootstrap iterations) are indicated in bold.

Population genetic structure Based on the discriminant analysis of principal components (DAPC), three genetic clusters were considered optimal to describe the data (lowest BIC value, see Fig. S2, Supplementary Material). Based on the two retained discriminant functions, the derived probabilities of group membership for each individual

were high (> 0.95 for each cluster). The three genetic clusters were not geographically restricted (Fig. 2), thus indicating great levels of admixture. Genetic cluster 2 was the most represented gene pool in the island populations, while genetic cluster 3 was the most represented one in the mainland populations. Only in Gran Canaria 1 cluster 3 was missing.

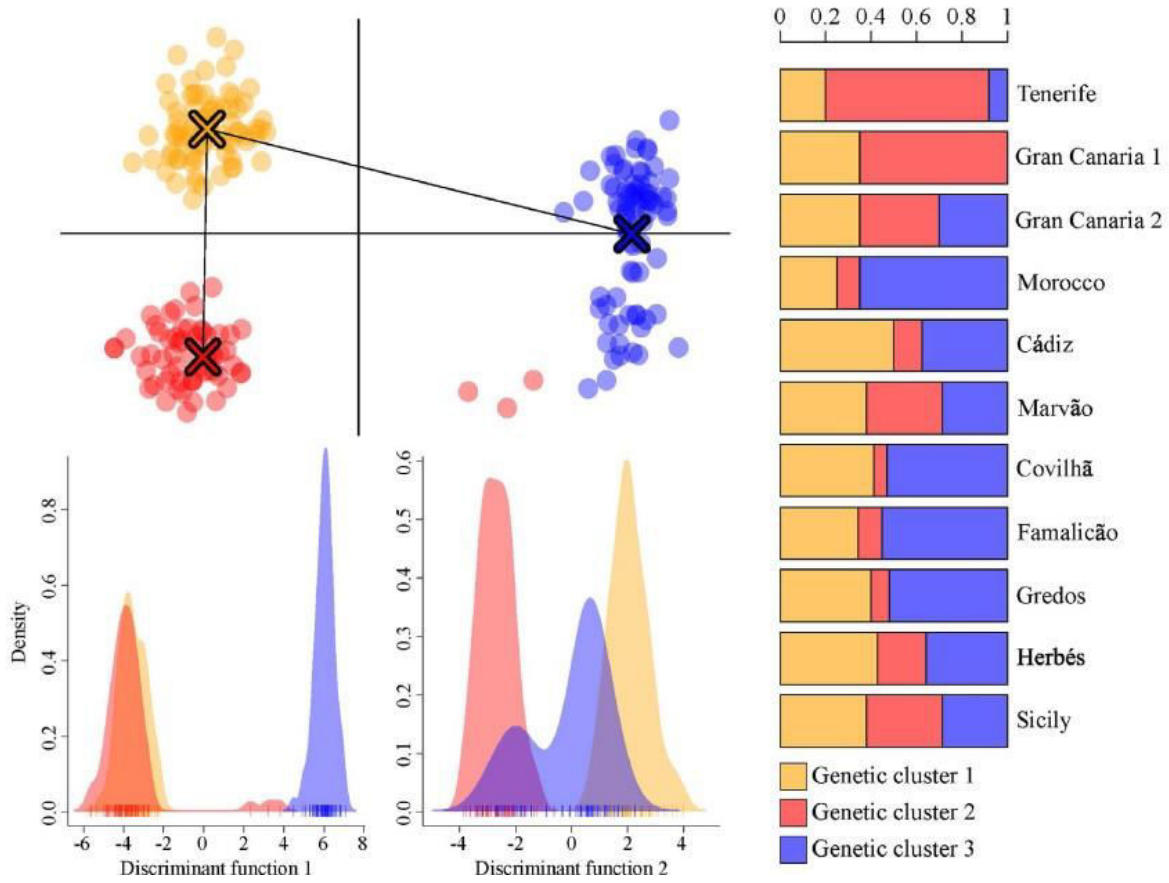

Figure 2 Scatter-plot (top left) of the three genetic clusters of *P. carporrhizans* resulting from discriminant analysis of principal components (DAPC). Black lines represent the minimum spanning tree based on the squared distances between clusters within the entire space. Bottom left: plot of the densities of individuals on the two retained discriminant functions. Right: stacked barplot of cluster distribution for 11 populations of *P. carporrhizans*.

Spatial analysis The isolation by distance analysis revealed a clear isolation by distance pattern (r = 0.472, *P* = 0.005). The scatter plot of local densities of distances (Fig. S3, Supplementary Material) showed a single consistent cloud of points indicating a continuous clines of genetic differentiation.

We found 29 centroids of microsatellite alleles (13.18% of all alleles) that were significantly geographically restricted (*P* < 0.05; Table S2, Supplementary Material).

The overall observed mean geographic distance between all shared alleles was 936.2 km and was significantly smaller from the expected mean under the assumption of panmixia (1,055.6 km, $P < 0.001$). The overall observed mean distance between geographically restricted alleles was also significantly smaller from the expected mean (956.5 vs 1,102.762 km), but higher than the overall observed mean geographic distance between all shared alleles thus indicating over-dispersion (Fig. S4).

Gene flow analysis We estimated the effective population size and migration rates with MIGRATE-N. The 191 effective population size (θ) of the Macaronesian islands group (1.16) was considerably 192 smaller than that of the mainland group (16.6). The migration analysis detected high, unidirectional gene flow from the mainland group to Macaronesia (Table S3, Supplementary Material).

Discussion

The Mediterranean Sea became separated from the Atlantic Ocean during the Messinian salinity crisis, approximately 5.5My ago (Garcia-Castellanos *et al.* 2009). While the patterns of genetic isolation between these two basins have been well documented in animals and plants (reviewed in Patarnello *et al.* 2007; Mairal *et al.* 2015; Warren *et al.* 2015), very few investigations have been conducted on the genetic diversity of lichen forming fungi between Macaronesian and Mediterranean populations (Widmer *et al.* 2012; Nuñez-Zapata *et al.* 2015). In the present study we analyzed the population genetic structure of the strictly outcrossing lichen forming fungus *P. carporrhizans* in the Mediterranean region. Sexually reproducing fungal species are expected to harbor higher genetic diversity but lower populationstructure than vegetatively reproducing species (Seymour *et al*. 2005; Werth 2010). Our results corroborate this prediction. Using highly variable microsatellite markers we showed that natural populations of *P. carporrhizans* exhibit high levels of genetic diversity and a complete absence of clonality. This is strikingly different from the trend reported for predominantly asexual lichen species, e.g. *L. pulmonaria*, whose populations harbor, almost always, a high number of clonal multilocus genotypes (Dal Grande *et al.* 2012; Widmer *et al.* 2012).

Allelic richness was comparatively lower in the peripheral populations of the Macaronesian islands. Macaronesian populations also displayed smaller effective population sizes. These are typical footprints of the so-called founder effect, i.e. the establishment of a new population from a small number of colonizing individuals

(Greenbaum *et al.* 2014). Founder events are often followed by a loss of alleles because founders seldom carry all the alleles of the ancestral population. Our results support therefore the 'abundant center' model of population dynamics (Vucetich & Waite 2003; Eckert *et al.* 2008) in *P. carporrhizans*, moving from highly diverse and core populations of the Mediterranean basin towards small and less dense populations at the edge of the species range.

Our analysis suggests an almost complete lack of population differentiation in *P. carporrhizans*. We found evidence for the existence of three genetic lineages with no clear geographic structure. In fact all lineages were present in similar frequencies in all investigated regions. As corroborated by the significant isolation by distance, the lack of genetic structure is largely a result of long distance dispersal connecting populations that are continuously distributed across the landscape. The small ascospores of *P. carporrhizans* (9.92 × 6.09 µm Argüello *et al.* 2007), which could be easily dispersed by wind, may explain the presence of shared alleles even at distances greater than 900 km.

In our study we did not find any evidence for the presence of barriers to dispersal. The absence of dispersal barriers facilitates long distance dispersal thus promoting the disruption of geographic structure in *P. carporrhizans* populations. Furthermore, *P. carporrhizans* inhabits areas such as the Mediterranean basin and Macaronesian islands, which are regions that escaped the past glacial events (Nieto-Feliner 2011). These regions have been suggested to be the glacial refugia for the common phorophytes of *P. carporrhizans*, i.e. *Quercus* spp (Gömöry *et al.* 2001; Atkinson *et al.* 2007; Neiva *et al.* 2012). Repeated glaciation cycles have been shown to cause isolation of populations, followed by speciation (Hewitt 2000). Escaping the glaciation events would thus have promoted population homogeneity and reduced population subdivision in *P. carporrhizans*.

Another species, for which the genetic variability and population structure has been analyzed using SSR markers, is *Buellia frigida* (Jones *et al.* 2015). In contrast to *P. carporrhizans*, *Buellia frigida* populations have comparatively higher genetic structure along with high genetic diversity. One of the reasons behind the higher population structure in *B. frigida* was suggested to be the limited establishment capability of the spores due to harsh environmental conditions in the Antarctica, which hinders inter population gene flow thus causing population differentiation (Jones *et al.* 2015). Moreover, the reproductive mode of *B. frigida* (self-fertile/homothallic species) is different from *P. carporrhizans,* which might also have contributed to the differences in population structures of the two species. As compared to vegetatively reproducing species however,

as expected, *P. carporrhizans* displayed lower genetic structure. For example, a recent study based on sequencing data, showed high population differentiation in the closely-related isidiate species, Parmelina *tiliacea* (Núñez-Zapata *et al.* 2015). Interestingly, unlike *P. carporrhizans*, the Macaronesian populations of *P. tiliacea* were the most genetically diverse.

The high genetic diversity of *P. carporrhizans* indicates high frequency of sexual recombination resulting in novel genotypic combinations at each generation. We obtained evidence for this prediction by analyzing the distribution of mating-type idiomorphs in *P. carporrhizans* populations.

Our results demonstrate that *P. carporrhizans* is a heterothallic, i.e. self incompatible, species. Moreover our data suggest that recombination is a common event in natural populations of this species as i) we retrieved both mating types in all studied populations, ii) even in populations with significantly skewed mating-type frequencies, values of linkage disequilibrium were close to zero (i.e., fully recombining populations) and clonality was absent. These results confirm the hypothesis put forward by Singh *et al.*38 for which mating-type imbalance cannot be used to predict clonality in populations of lichen-forming fungi. MAT ratios depend in fact upon population history and founder individuals MAT genes. The slightly higher linkage values obtained for some populations may thus be the result of spatial heterogeneity and/or of an origin from a small number of parental thalli. In the case of Cádiz, instead, the significant linkage disequilibrium may simply be an artifact of the limited sample size. In conclusion, the presence of both mating types in each *P. carporrhizans* population seems to be sufficient to ensure panmictic populations and, thus, low levels of linkage disequilibrium.

Genetic diversity and population structure analyses of *P. carporrhizans* suggested that core populations of the Mediterranean basin and edge populations in the Macaronesian islands are still highly connected via gene flow. To corroborate this hypothesis, we estimated migration rates between these two population groups. Our results indicate that populations across the southern species range are still highly connected. However, we found that gene flow is unidirectional, that is, from core towards edge populations. This may well explain the smaller population size and lower allelic richness of the island populations. Moreover, on the Canary Islands, *P. carporrhizans* does not grow at low altitudes, where it is replaced mainly by *Parmotrema* species, but grows in the Erica–Myrica heath ('fayal-brezal') communities on well-lit borders of the laurel forest ('monteverde'), on sweet chestnut, elms, poplars, pines, etc. from 800 to 1500 m a.s.l. The

composition of the forests (at least in Tenerife) has undergone major changes during the Late Holocene, in particular, suitable habitats such as arboreal species of the vegetation alongside laurel forest have considerably declined as a consequence of human activity and climatic variation (Nascimento *et al.* 2009). On the basis of the limited patches of suitable habitat and the impoverished genetic make-up of the local populations, it can be predicted that island populations are destined to serve only as sink populations.

Based on these observations, we propose that migratory fluxes from core populations can only partially compensate for the detrimental effects of drift, likely caused by founder effects, by maintaining relatively high genetic diversity in peripheral populations.

Several studies on different organisms (e.g.Kirkpatrick & Ravigne 2002; Vucetich & Waite 2003; Havrdová *et al.* 2015; Volis *et al.* 2016) have shown that peripheral populations that are migration dependent may exhibit lower diversity and smaller population sizes than core populations. Therefore, migration-dependent peripheral populations have been shown to be more vulnerable to the detrimental effects of genetic drift (e.g.Pujoll & Pannell 2008), a process so-called extinction-vortex (Gilpin *et al.* 1986). Our findings of almost unidirectional migration and lower population size of the edge populations is in accord with the traditional view of islands as the end of the colonization road (Bellemain & Ricklefts 2008). The lack of one of the three genetic lineages in a population of the Canary Islands further corroborates this hypothesis. Future studies including additional peripheral populations, in particular the locally endangered populations of central and northern Europe, would provide additional evidence for the proposed scenario.

Conclusions

In conclusion, we showed that the strictly sexual, heterothallic lichen-forming fungus *P. carphorrizans* harbors high genetic diversity and connectivity across the Mediterranean region. As a result, we did not find evidence for population structure, suggesting that populations of *P. carphorrizans* act as components of a metapopulation system highly connected by gene flow. The genetic composition of *P. carporrhizans* can thus be explained by a core-edge population model with high migration rates from the Mediterranean basin (in particular the Iberian Peninsula) towards the Macaronesian islands.

The apparent absence of dispersal barriers found in our study implies that the establishment of newly dispersed fungal spores is not limited by the availability of

compatible photobionts (Werth *et al.* 2006). This, combined with high levels of mycobiont diversity and of recombination in populations, leads to the expectation of high local availability and high levels of horizontal transmission of the photosynthetic partners (Dal Grande *et al.* 2012). As a result, one would expect incongruent fungal-algal population structures (Yahr *et al.* 2006; Fernández-Mendoza *et al.* 2011; Widmer *et al.* 2012). *Parmelina* carporrhizans represents an ideal system to test these predictions for strictly outcrossing lichen-forming fungi. Additional studies in this direction can further shed light on the effect of dispersal mode on the co-phylogeographic dynamics of the lichen symbiosis.

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Author contributions

DA, PKD and AC conceived and designed the study. DA and FDG performed the experiments; DA, FDG and PC analyzed the data. MCM and IS contributed reagents/materials. AC, DA, PKD and PC collected the samples from the field. DA and FDG wrote the paper; all authors discussed the results and revised the manuscript. AC approved the final manuscript.

Supplementary Material

Supplementary Table 1: Voucher information and multilocus genotypes (8 microsatellite markers) for 220 *P. carporrhizans* thalli from 11 populations.

Population	DNA	Pcar1	Pcar4	Pcar2	Pcar3	Pcar5	Pcar8	Pcar6	Pcar7	Longitude	Latitude	Altitude	Phorophyte
Tenerife	3678*	137	295	264	188	229	427	213	145	-16.4153	28.4533	894	*Castanea sativa*
Tenerife	3679*	137	315	262	239	249	371	213	205	-16.4153	28.4533	894	*Castanea sativa*
Tenerife	3680*	137	290	264	182	243	432	219	155	-16.4153	28.4533	894	*Castanea sativa*
Tenerife	3681*	137	290	262	188	227	432	219	174	-16.4153	28.4533	894	*Castanea sativa*
Tenerife	3682*	123	300	262	239	272	432	219	202	-16.4153	28.4533	894	*Castanea sativa*
Tenerife	3683*	147	310	264	212	272	432	219	202	-16.4153	28.4533	894	*Castanea sativa*
Tenerife	3684*	135	320	262	191	262	371	216	211	-16.4153	28.4533	894	*Castanea sativa*
Tenerife	3685*	137	295	262	182	-	432	219	145	-16.4153	28.4533	894	*Castanea sativa*
Tenerife	3686*	137	295	264	191	272	432	207	211	-16.4153	28.4533	894	*Castanea sativa*
Tenerife	3687*	137	290	264	236	272	371	213	211	-16.4153	28.4533	894	*Castanea sativa*
Tenerife	3688	139	310	248	197	229	432	213	148	-16.4153	28.4533	894	*Castanea sativa*
Tenerife	3689	137	315	260	191	229	381	219	223	-16.4153	28.4533	894	*Castanea sativa*
Tenerife	3690	137	290	262	239	229	381	219	148	-16.4153	28.4533	894	*Castanea sativa*
Tenerife	4369*	137	290	248	185	249	432	219	197	-16.4154	28.4513	895	*Prunus cerasus*
Tenerife	4370	137	290	248	239	243	432	216	205	-16.4154	28.4513	895	*Prunus cerasus*
Tenerife	4371	137	290	260	191	249	435	219	216	-16.4154	28.4513	895	*Prunus cerasus*
Tenerife	4372	137	315	262	185	229	381	251	205	-16.4154	28.4513	895	*Prunus cerasus*
Tenerife	4373	137	315	262	239	243	371	219	171	-16.4154	28.4513	895	*Prunus cerasus*
Tenerife	4374	137	315	262	185	229	381	251	205	-16.4154	28.4513	895	*Prunus cerasus*
Tenerife	4375	137	295	262	236	243	371	270	148	-16.4154	28.4513	895	*Prunus cerasus*
Tenerife	4376	137	315	260	191	229	432	219	148	-16.4154	28.4513	895	*Prunus cerasus*
Tenerife	4377	137	315	248	230	249	432	216	171	-16.4154	28.4513	895	*Prunus cerasus*
Tenerife	4378*	141	290	248	239	229	371	216	123	-16.4154	28.4513	895	*Prunus cerasus*
Tenerife	4379	137	275	260	185	227	432	270	123	-16.4154	28.4513	895	*Prunus cerasus*
Tenerife	4380	137	295	248	185	229	435	219	148	-16.4154	28.4513	895	*Prunus cerasus*
Gran Canaria1	3645*	137	300	264	185	243	393	219	185	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3646*	137	295	250	236	229	371	219	185	-15.5925	27.9892	1499	*Pinus radiata*
Gran Canaria1	3647*	137	280	250	236	227	417	219	168	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3648*	137	310	262	233	249	414	261	168	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3649*	141	295	262	182	227	-	204	120	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3650*	137	305	264	212	251	396	246	165	-15.5925	27.9892	1552	*Pinus radiata*

Gran Canaria1	3651*	137	305	264	221	229	396	219	165	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3652*	137	315	250	224	229	417	213	120	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3653*	141	295	250	236	229	417	213	120	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3654*	139	295	250	236	243	408	207	202	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3655*	137	295	262	191	241	371	213	202	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3656*	123	290	264	191	229	408	210	223	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3657*	137	295	262	212	229	408	213	202	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3658*	123	295	250	236	229	371	270	202	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3659*	137	295	250	236	229	432	213	202	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3660*	139	315	264	236	229	432	246	220	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3661*	137	280	264	233	229	432	204	220	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3662*	137	295	262	248	249	393	261	220	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3663*	137	315	262	182	229	371	261	145	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria1	3664*	141	290	206	242	249	432	261	211	-15.5925	27.9892	1552	*Pinus radiata*
Gran Canaria2	3619	135	295	262	212	229	371	213	214	-15.5414	28.0003	945	*Prunus cerasus*
Gran Canaria2	3620	123	265	262	185	243	432	225	177	-15.5414	28.0003	945	*Prunus cerasus*
Gran Canaria2	3621	141	300	262	236	229	393	219	205	-15.5414	28.0003	945	*Prunus cerasus*
Gran Canaria2	3622	137	275	262	242	241	393	219	148	-15.5414	28.0003	945	*Prunus cerasus*
Gran Canaria2	3623	123	275	260	191	249	371	219	123	-15.5414	28.0003	945	NA
Gran Canaria2	3624	137	315	248	212	241	420	213	-	-15.5414	28.0003	945	NA
Gran Canaria2	3625	123	295	248	236	229	371	216	211	-15.5414	28.0003	945	*Prunus cerasus*
Gran Canaria2	3626	137	315	262	239	227	432	213	148	-15.5414	28.0003	945	*Prunus cerasus*
Gran Canaria2	3627	137	280	262	239	229	393	-	205	-15.5414	28.0003	945	NA
Gran Canaria2	3628*	123	295	262	224	229	371	261	171	-15.5414	28.0003	945	*Prunus cerasus*
Gran Canaria2	3629	123	295	260	185	227	371	213	182	-15.5414	28.0003	945	NA
Gran Canaria2	3630	123	315	260	194	249	432	225	223	-15.5414	28.0003	945	NA
Gran Canaria2	3631	137	295	260	236	229	417	207	205	-15.5414	28.0003	945	*Chamaecytisus proliferus*
Gran Canaria2	3636	123	-	262	-	249	420	246	-	-15.5414	28.0003	945	NA
Gran Canaria2	3637	137	320	248	236	227	408	261	123	-15.5414	28.0003	1329	*Ulmus sp.*
Gran Canaria2	3638*	123	320	262	236	249	420	246	205	-15.5414	28.0003	1329	*Ulmus sp.*
Gran Canaria2	3639	123	290	260	242	227	427	213	171	-15.5414	28.0003	1329	NA
Gran Canaria2	3641	123	280	260	200	216	432	219	225	-15.5414	28.0003	1329	NA
Gran Canaria2	3642	139	275	260	-	-	-	216	171	-15.5414	28.0003	1329	NA
Gran Canaria2	3668	137	330	262	230	229	374	213	223	-15.5414	28.0003	1329	NA
Morocco	4100	135	305	260	191	245	414	213	-	-5.3742	35.3460	701	*Olea europaea*
Morocco	4101	123	305	262	176	-	466	219	-	-5.3742	35.3460	701	*Olea europaea*

Morocco	4102	123	300	248	200	245	414	216	168	-5.3742	35.3460	701	*Olea europaea*
Morocco	4103	123	300	260	239	229	408	213	157	-5.3742	35.3460	701	NA
Morocco	4104	123	305	262	176	243	466	219	168	-5.3742	35.3460	701	*Olea europaea*
Morocco	4105	123	285	260	188	243	396	210	168	-5.3742	35.3460	701	*Olea europaea*
Morocco	4106	145	300	260	176	225	414	210	254	-5.3742	35.3460	701	NA
Morocco	4107	129	310	260	270	253	417	207	216	-5.3742	35.3460	701	NA
Morocco	4108	123	285	260	153	229	420	219	197	-5.3873	35.3504	655	*Pinus sp.*
Morocco	4109	123	290	260	182	251	414	249	223	-5.3873	35.3504	655	*Pinus sp.*
Morocco	4110	137	250	260	224	229	406	197	155	-5.3873	35.3504	655	*Pinus sp.*
Morocco	4111	137	300	262	236	225	388	186	123	-5.3873	35.3504	655	*Pinus sp.*
Morocco	4112	141	305	260	233	229	381	179	214	-5.3873	35.3504	655	*Pinus sp.*
Morocco	4114	135	325	260	194	249	411	219	216	-5.3873	35.3504	655	*Pinus sp.*
Morocco	4118	137	275	260	167	251	406	213	234	-5.3873	35.3504	655	*Pinus sp.*
Morocco	4119	123	295	248	191	249	452	219	228	-5.3873	35.3504	655	*Pinus sp.*
Morocco	4120*	123	295	260	194	249	399	219	197	-5.3873	35.3504	655	*Pinus sp.*
Morocco	4122	137	285	262	230	256	411	179	171	-5.3873	35.3504	655	*Pinus sp.*
Morocco	4123	137	290	260	170	249	399	222	182	-5.3873	35.3504	655	*Pinus sp.*
Morocco	4124	123	290	262	251	229	399	207	125	-5.3873	35.3504	655	*Pinus sp.*
Cádiz	4384	123	295	262	191	251	-	-	165	-5.3776	36.7828	1168	*Pinus sp.*
Cádiz	4385	-	285	260	150	229	396	210	182	-5.3776	36.7828	1168	*Pinus sp.*
Cádiz	4387	-	295	262	191	251	359	210	165	-5.3776	36.7828	1168	*Pinus sp.*
Cádiz	4388	141	300	262	182	243	396	179	208	-5.3776	36.7828	1168	*Pinus sp.*
Cádiz	4389*	123	310	240	221	229	399	204	202	-5.3776	36.7828	1168	*Pinus sp.*
Cádiz	4390	137	305	250	188	243	381	197	157	-5.3776	36.7828	1168	*Pinus sp.*
Cádiz	4392*	127	285	260	150	229	381	210	194	-5.3776	36.7828	1168	*Pinus sp.*
Cádiz	4421	127	285	260	153	229	396	210	182	-5.3776	36.7828	1168	*Pinus sp.*
Marvão	4492	123	310	260	197	251	396	216	165	-7.3785	39.3951	858	*Castanea sativa*
Marvão	4493	137	310	262	173	251	393	222	145	-7.3785	39.3951	858	*Castanea sativa*
Marvão	4494	143	300	262	167	251	417	173	-	-7.3785	39.3951	858	*Castanea sativa*
Marvão	4495	141	320	262	173	249	404	213	162	-7.3785	39.3951	858	*Castanea sativa*
Marvão	4496*	137	290	260	182	249	427	216	165	-7.3785	39.3951	858	*Castanea sativa*
Marvão	4497	137	275	284	206	243	396	216	115	-7.3785	39.3951	858	*Castanea sativa*
Marvão	4498	137	280	248	176	251	396	279	188	-7.3785	39.3951	858	*Castanea sativa*
Marvão	4499	133	300	262	212	229	396	210	157	-7.3785	39.3951	858	*Castanea sativa*
Marvão	4500	135	285	260	188	243	411	207	177	-7.3785	39.3951	858	*Castanea sativa*
Marvão	4501	157	260	262	197	251	441	184	208	-7.3785	39.3951	858	*Castanea sativa*

Marvão	4502	137	320	262	170	227	406	210	162	-7.3785	39.3951	858	*Castanea sativa*
Marvão	4503	137	325	248	194	251	408	207	157	-7.3785	39.3951	858	*Castanea sativa*
Marvão	4504	137	240	266	209	243	393	210	125	-7.3788	39.3995	711	*Ulmus sp.*
Marvão	4505	143	290	262	221	251	420	194	-	-7.3788	39.3995	711	*Ulmus sp.*
Marvão	4506	123	310	262	185	251	374	213	145	-7.3788	39.3995	711	*Ulmus sp.*
Marvão	4507	137	305	260	185	253	439	216	123	-7.3788	39.3995	711	*Ulmus sp.*
Marvão	4508*	139	310	260	197	249	-	225	162	-7.3788	39.3995	711	*Ulmus sp.*
Marvão	4509	123	320	262	191	249	-	207	162	-7.3788	39.3995	711	*Ulmus sp.*
Marvão	4510	141	290	266	230	251	396	219	125	-7.3788	39.3995	711	*Ulmus sp.*
Marvão	4511	137	275	260	284	227	411	216	157	-7.3788	39.3995	711	*Ulmus sp.*
Marvão	4512	137	305	262	254	243	-	216	145	-7.3788	39.3995	711	*Ulmus sp.*
Covilhã	4513	123	310	260	176	243	-	204	185	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4514	137	315	260	185	251	439	216	159	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4515	-	330	262	206	249	399	279	157	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4516*	141	300	260	197	243	-	204	157	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4517	143	330	242	185	253	396	179	188	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4518	123	275	280	185	253	414	204	188	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4519	141	295	260	203	251	406	207	191	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4520	141	325	260	191	243	402	204	125	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4521	123	300	260	236	225	406	216	159	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4522*	135	290	260	191	251	411	204	157	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4523	139	315	282	170	249	411	222	174	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4524	123	320	266	197	229	406	204	155	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4525	253	290	260	185	253	417	279	314	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4526	123	310	272	167	227	399	216	151	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4527	123	310	268	185	251	396	207	251	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4528	137	290	262	185	229	-	222	162	-7.5236	40.2863	990	*Quercus robur*
Covilhã	4529	137	250	260	188	253	383	210	112	-7.5236	40.2863	990	*Quercus robur*
Famalicão	4530	123	270	260	188	249	-	216	151	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4531	137	250	260	197	229	-	207	145	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4532*	127	300	248	182	247	396	197	237	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4533	135	300	262	206	229	408	213	145	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4534	123	300	258	200	256	-	186	110	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4535	137	320	282	218	249	-	204	157	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4536	141	305	260	200	227	-	210	157	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4537	137	310	244	185	229	396	207	162	-7.3697	40.4463	817	*Quercus sp.*

Famalicão	4538	141	305	260	153	247	404	207	123	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4539*	137	245	248	197	256	393	210	113	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4540	127	330	260	188	259	-	207	157	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4541	123	265	262	182	-	385	-	-	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4542	139	250	262	185	227	359	-	-	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4543	153	285	248	203	251	-	210	145	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4544	123	310	260	194	253	359	207	165	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4545	133	300	266	179	249	408	191	159	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4546	137	320	260	215	227	365	207	145	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4547	123	290	260	191	253	371	207	162	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4548	123	290	262	185	227	414	213	162	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4549	123	265	260	203	249	408	191	194	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4550	164	305	260	209	243	460	207	191	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4551	123	270	260	215	251	406	216	157	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4552	141	325	260	188	249	-	197	145	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4554	145	305	260	218	249	327	232	157	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4555	137	290	260	206	229	292	207	145	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4556	-	315	266	194	259	359	216	113	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4557	137	305	260	197	227	406	207	157	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4558	143	310	266	194	243	411	213	157	-7.3697	40.4463	817	*Quercus sp.*
Famalicão	4559	135	305	260	167	243	323	191	137	-7.3697	40.4463	817	*Quercus sp.*
Gredos	3350	123	240	260	248	256	437	204	113	-5.0124	40.3234	1363	*Ulmus sp.*
Gredos	3364	141	295	294	164	251	388	207	223	-5.0124	40.3234	1363	*Ulmus sp.*
Gredos	3365	137	270	260	191	243	460	207	162	-5.0124	40.3234	1363	*Ulmus sp.*
Gredos	3366	133	250	262	212	253	399	270	123	-5.0124	40.3234	1363	*Ulmus sp.*
Gredos	3367	123	280	260	203	249	402	184	214	-5.0124	40.3234	1363	*Ulmus sp.*
Gredos	3418	123	325	258	194	229	443	207	125	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3419	137	235	248	179	251	417	204	137	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3420*	141	260	274	227	249	402	207	157	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3421	137	290	260	212	251	406	210	216	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3422*	141	290	260	212	251	408	204	162	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3423	123	305	242	233	251	390	289	157	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3424	141	295	260	182	243	414	204	157	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3425	141	290	260	212	259	396	216	197	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3426	137	300	260	191	251	427	204	145	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3427	123	270	260	182	239	441	219	145	-5.0110	40.3079	1363	*Castanea sativa*

Gredos	3428	123	250	262	200	251	404	213	185	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3429	139	280	252	215	249	393	216	168	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3430	123	305	260	239	233	396	194	148	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3431	143	250	262	188	251	411	207	157	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3432	141	315	230	179	229	385	216	245	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3433	137	300	248	242	229	399	228	148	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3434	127	295	260	161	247	408	197	142	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3435	127	255	260	215	229	396	179	177	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3436	143	275	248	182	249	381	173	208	-5.0110	40.3079	1363	*Castanea sativa*
Gredos	3437	123	313	260	191	251	404	179	223	-5.0110	40.3079	1363	*Castanea sativa*
Herbés	4398	137	265	260	218	251	388	213	-	-0.0227	40.6899	919	*Quercus faginea*
Herbés	4399	137	265	260	218	251	388	213	205	-0.0227	40.6899	919	*Quercus faginea*
Herbés	4400*	141	285	248	221	239	404	170	225	-0.0227	40.6899	919	*Quercus faginea*
Herbés	4401	143	280	274	209	251	423	207	157	-0.0227	40.6899	919	*Quercus faginea*
Herbés	4405	135	295	260	218	247	408	216	237	-0.0227	40.6899	919	*Quercus faginea*
Herbés	4406*	135	285	242	224	229	396	204	223	-0.0227	40.6899	919	*Quercus faginea*
Herbés	4411	137	290	248	179	229	411	219	151	-0.0227	40.6899	919	*Quercus faginea*
Herbés	4416	141	320	262	188	241	414	182	159	-0.0227	40.6899	919	*Quercus faginea*
Herbés	4417	137	300	260	179	229	396	210	182	-0.0227	40.6899	919	*Quercus faginea*
Herbés	4690	135	305	-	-	251	396	179	-	-0.0227	40.6899	919	*Quercus faginea*
Herbés	4691*	137	305	248	194	249	411	270	216	-0.0227	40.6899	919	*Quercus faginea*
Herbés	4694	-	305	262	209	243	399	182	174	-0.0227	40.6899	919	*Quercus faginea*
Herbés	4695	-	335	260	194	251	408	207	145	-0.0227	40.6899	919	*Quercus faginea*
Herbés	4696	137	330	262	206	229	393	219	148	-0.0227	40.6899	919	*Quercus faginea*
Sicily	3983	123	-	248	203	241	404	207	194	14.6271	37.9451	1183	*Quercus sp.*
Sicily	3984	127	290	266	221	241	414	160	165	14.6271	37.9451	1183	*Quercus sp.*
Sicily	3985	125	-	260	200	251	-	207	165	14.6271	37.9451	1183	*Quercus sp.*
Sicily	4011	137	260	260	197	239	399	261	265	14.4889	37.8817	1223	*Quercus sp.*
Sicily	4012	137	295	262	203	251	-	194	205	14.4889	37.8817	1223	*Quercus sp.*
Sicily	4013	133	285	248	197	249	-	207	139	14.4889	37.8817	1223	*Quercus sp.*
Sicily	4014	137	315	262	197	241	-	207	159	14.4889	37.8817	1223	*Quercus sp.*
Sicily	4015	141	320	248	194	229	381	191	162	14.4889	37.8817	1223	*Quercus sp.*
Sicily	4016	137	320	268	194	249	443	204	191	14.4889	37.8817	1223	*Quercus sp.*
Sicily	4053	141	305	260	185	251	396	179	274	13.3863	37.8685	872	*Quercus sp.*
Sicily	4054	123	260	260	197	243	-	210	205	13.3863	37.8685	872	*Quercus sp.*
Sicily	4055	141	320	260	194	251	399	210	157	13.3863	37.8685	872	*Quercus sp.*

Sicily	4056	143	295	250	206	243	378	207	162	13.3863	37.8685	872	*Quercus sp.*
Sicily	4057	141	290	248	215	239	393	160	157	13.3863	37.8685	872	*Quercus sp.*
Sicily	4058	135	250	262	209	229	402	216	123	13.3863	37.8685	872	*Quercus sp.*
Sicily	4059	137	280	248	200	249	414	207	159	13.3863	37.8685	872	*Quercus sp.*
Sicily	4060	137	275	262	185	249	414	201	194	13.3863	37.8685	872	*Quercus sp.*
Sicily	4061	137	325	294	218	229	383	216	188	13.3863	37.8685	872	*Quercus sp.*
Sicily	4062	135	250	242	206	245	396	216	165	13.3863	37.8685	872	*Quercus sp.*
Sicily	4063	137	250	262	155	251	396	204	115	13.3863	37.8685	872	*Quercus sp.*
Sicily	4064	129	310	260	194	233	393	207	113	13.3863	37.8685	872	*Quercus sp.*
Number of alleles/locus	-	16	22	20	36	17	35	28	46	-	-	-	-

Supplementary table details of the specimens kept in MAF Herbarium (Madrid, Spain)

Gran Canaria 2: DNA 3638(MAF-Lich 20664) and 3628 (MAF-Lich 20665) Spain, Gran Canaria, San Mateo, A. Crespo, P. Cubas, A. Santos and P.K. Divakar in 23/06/2009. **Tenerife:** DNA 4378(MAF-Lich 20666) and 4369(MAF-Lich 20667) Spain, Tenerife, Sauzal A. Crespo, P. Crespo and V. J. Rico in 14/03/2014. **Morocco:** DNA 4120(MAF-Lich 20668) Morocco, Tetuán, Beni Hassan, D. Alors and C.G. Boluda in 21/10/2013. **Cadiz**: DNA 4389(MAF-Lich 20669) and 4392(MAF-Lich 20670) Spain, Cadiz, Grazalema A. Crespo, J. Fdez de Bobadilla and J. Nuñez-Zapata in 15/102010. **Marvão:** DNA 4508(MAF-Lich 20671) and 4496(MAF-Lich 20672) Portugal, Alto Alentejo, Marvão D. Alors, A. Crespo, P.K. Divakar, C. Ruibal and V. J. Rico in 11/06/2014. **Covilhã**: DNA 4516(MAF-Lich 20673) and 4522 (MAF-Lich 20674) Portugal, Beira Baixa, Covilhã D. Alors, A. Crespo, P.K. Divakar, C. Ruibal and V. J. Rico 12/06/2014. **Famalicão**: DNA 4539(MAF-Lich 20675) and 4532(MAF-Lich 20676) Portugal, Beira Baixa, Famalicão D. Alors, A. Crespo, P.K. Divakar, C. Ruibal and V. J. Rico in 13/06/2014. **Herbes:** DNA 4400(MAF-Lic 20677) and 4406(MAF-Lich 20679) Spain, Castellón, Herbés D. Alors and V. Claramonte† in 19/05/2014 and DNA 4691 (MAF-Lich 20678) D. Alors, C. Alors, J. Villagra and N. Perales in 29/12/2014.

***** DNA codes 3645 to 3664 (MAF-Lich 19125 to 19142), 3678 to 3687 (MAF-Lich 19143 to 19152) and 3420, 3422 (MAF-Lich 19191 and MAF-Lich 19192) were included in Alors *et al.* 2014 (Chapter IV)

Supplementary Table 2: Geographically restricted alleles of *Parmelina carporrhizans* and geographic coordinates of their centroids.

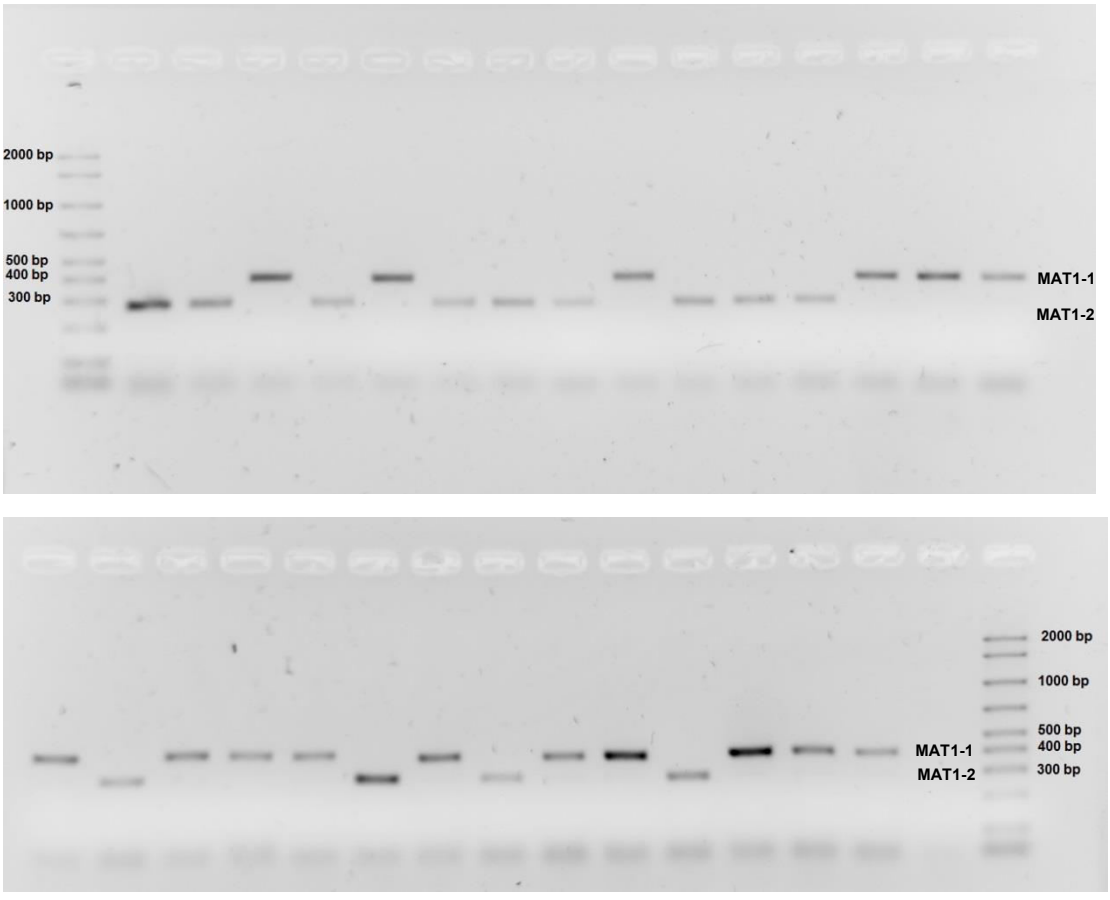
Locus	Alllele	Long	Lat
Pcar1	137	-8.63236	33.98925
Pcar4	250	-0.25257	39.10741
Pcar4	295	-10.54506	31.86994
Pcar4	315	-12.29158	31.46331
Pcar2	250	-11.23765	30.06395
Pcar2	264	-15.96650	28.20011
Pcar3	197	-1.45035	38.46678
Pcar3	203	0.30676	39.55485
Pcar3	206	-0.41309	39.57157
Pcar3	218	-0.23685	40.13847
Pcar3	236	-14.53727	29.27917
Pcar3	239	-13.99602	30.23680
Pcar5	229	-9.39365	33.39248
Pcar5	251	-3.18724	39.09053
Pcar5	272	-16.41531	28.45325
Pcar8	371	-15.37119	28.94487
Pcar8	396	-3.92974	38.19653
Pcar8	432	-16.07599	28.26934
Pcar6	207	-2.82068	38.42865
Pcar6	213	-11.34349	32.67785
Pcar6	219	-12.20842	31.28559
Pcar6	246	-15.56697	27.99472
Pcar7	120	-15.59250	27.98917
Pcar7	148	-12.32043	31.95665
Pcar7	159	0.90593	39.57651
Pcar7	171	-14.14040	29.37563
Pcar7	202	-14.52135	29.20439
Pcar7	211	-16.07597	28.26984
Pcar7	220	-15.59250	27.98917

Supplementary Table 3: MIGRATE-N Bayesian Analysis posterior distribution table.

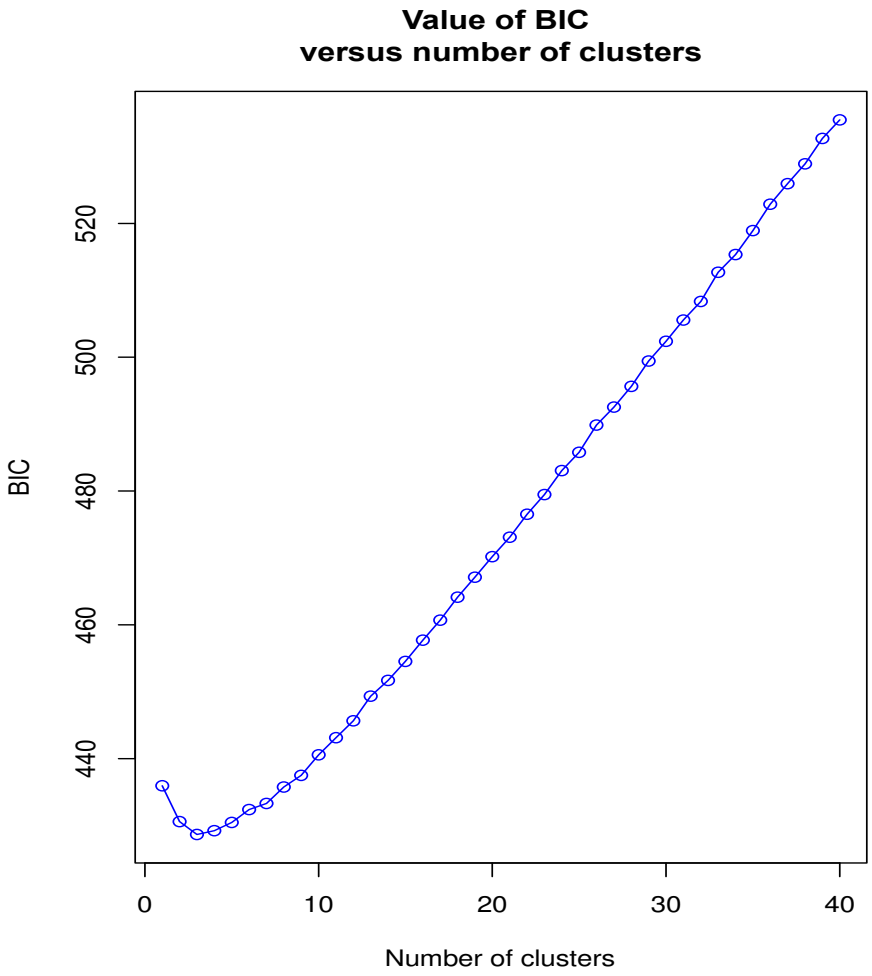
Parameter	2.5%	25.0%	75.0%	97.5%	Mean
Θ1	0.17	0.73	1.53	2.10	1.16
Θ2	9.13	11.70	15.90	21.33	16.59
M2->1	3.20	3.97	5.07	5.70	8.39
M1->2	0.03	0.53	1.30	1.83	0.94

1: Macaronesian Islands populations; 2: Mainland populations (Morocco, Iberian Peninsula, Sicily); Θ: mutation-scaled effective population size; M: mutation-scaled immigration rate.

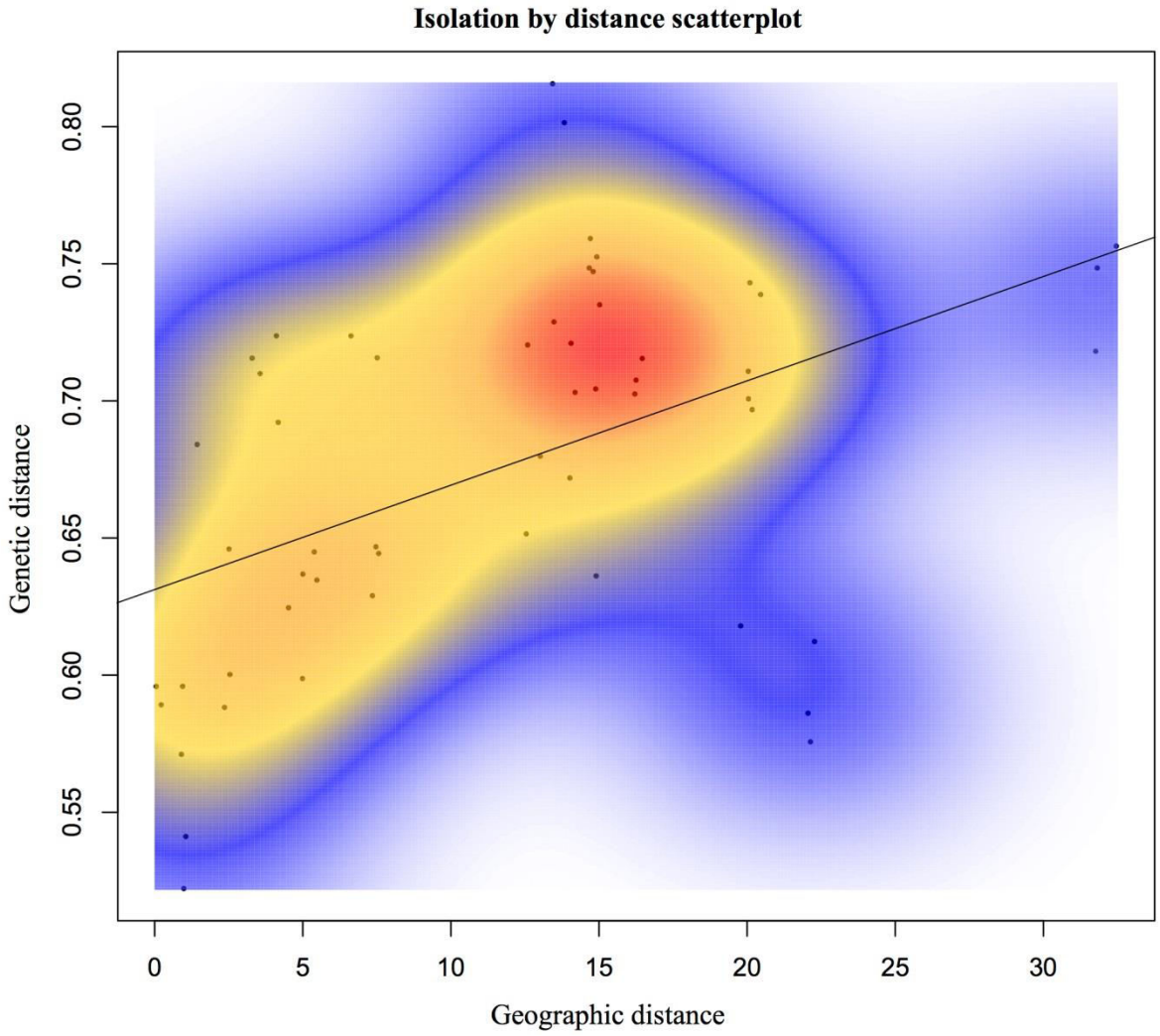
Supplementary Figure 1: An example of gel electrophoresis of the PCR products of the mating-type PCR analysis on 29 thalli of *P. carporrhizans* (Famalicão 4534-4559 and Gran Canaria2 3620, 3625-3626). Molecular ruler: AmpliSize 50–2,000 bp (BioRad). Stain: SYBR Safe (Invitrogen, USA).



Supplementary Figure 2: Inference of the number of clusters in the DAPC performed on 220 samples of *P. carporrhizans*. A K value of 3 (the lowest BIC value) represents the best summary of the data.

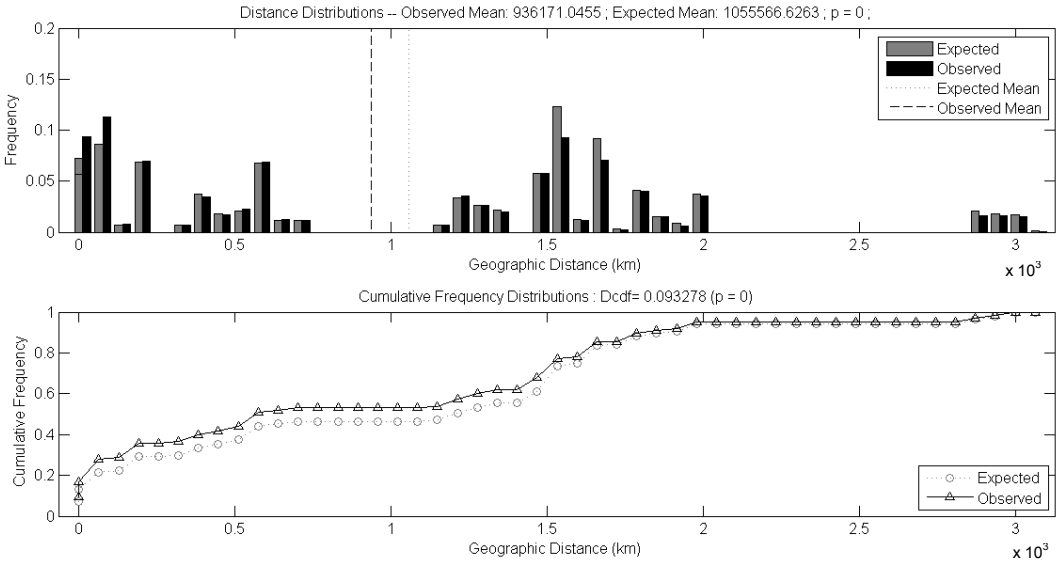


Supplementary Figure 3: Isolation by distance plot illustrating a continuous cline of genetic differentiation in *P. carporrhizans* populations.

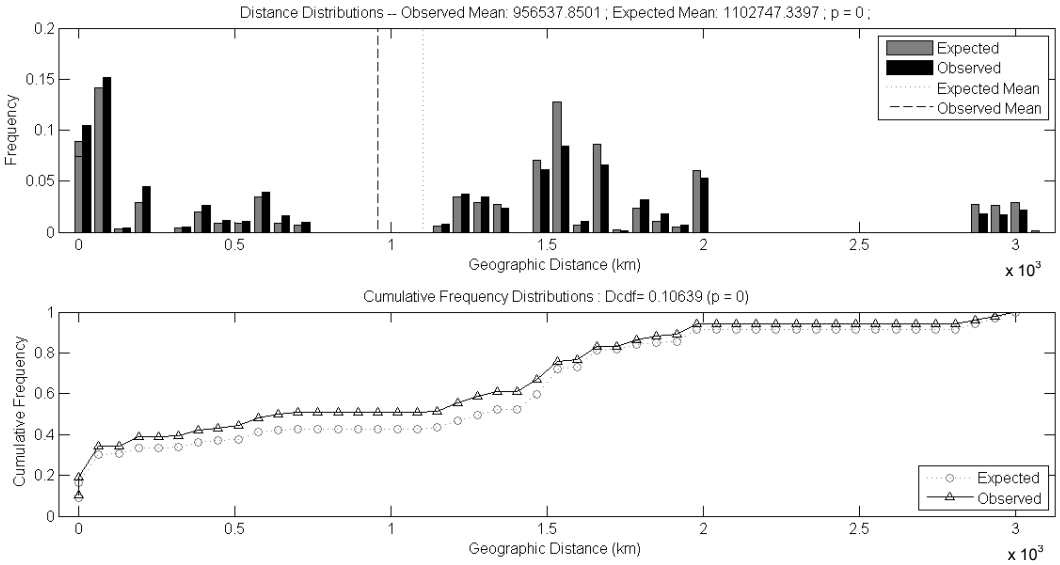


Supplementary Figure 4: Spatial distribution analysis of a) all, and b) geographically restricted shared alleles of *P. carporrhizans*.

a)



b)



DISCUSIÓN GENERAL

El estudio de la diversidad en líquenes pone de manifiesto que aún queda diversidad oculta por descubrir. Las especies de distribución disyunta, como *Punctelia rudecta* s. lat. pueden albergar múltiples especies crípticas geográficamente aisladas (Capítulo 1), como se ha visto en casos previos (*Parmelina quercina*, Argüello *et al.* 2007; revisión, Crespo & Perez-Ortega 2009; revisión, Crespo & Lumbsch 2010; *Melanelixia glabra*, Divakar *et al.* 2010. *Leptogium* Otálora *et al.* 2010; revisión, Lumbsch & Leavitt 2011; *Xanthoparmelia*, Amo de Paz *et al. 20*12; *Cladia aggregata* Parnmen *et al.* 2012; *Sticta,* Moncada *et al.* 2014; *Montanelia,* Leavitt *et al.* 2015). Igualmente hemos hallado variabilidad oculta en las poblaciones intratalinas de fotobiontes de *Anaptychia ciliaris* y *Xanthoria parietina*, que como hemos revelado tras el análisis con microsatélites albergan múltiples fotobiontes (Capítulo 2), indicando que la diversidad de los fotobiontes *Trebouxiodes* puede ser la norma general en lugar de excepciones o casos aislados (Casano *et al.* 2011; Mansournia *et al.* 2012, Muggia *et al.* 2013). La evaluación con los marcadores apropiados nos permite apreciar la variabilidad sutil y diferenciar entre genotipos relacionados, no relacionados y clonales. Los marcadores genéticos clásicos encuentran baja o nula variabilidad en el seno de las especies; sin embargo encontramos gran variabilidad alanalizarlos con marcadores altamente polimórficos (microsatélites), especialmente en especies de reproducción sexual (Capítulos 2, 4 y 5).

La geografía, en combinación con el modo de reproducción, tiene un gran peso en procesos de especiación y estructuración poblacional en líquenes. Y a la inversa, la geografía se convierte en un carácter diagnóstico para la determinación de las especies (Ej. Argüello *et al.* 2007; Divakar *et al.* 2010; Leavitt *et al.* 2015b; Kirika *et al.* 2016), como hemos visto en el capítulo 1. En las regiones aisladas geográficamente, como Macaronesia pueden darse fenómenos de diferenciación entre poblaciones, como el patrón de aislamiento por distancia (IBD) o el efecto fundador observado en las poblaciones de *Parmelina carporrhizans* (Capítulo 5). Incluso potencialmente pueden conducir a la formación de especies endémicas como *Punctelia guanchica* (Capítulo 1). El modo de reproducción va a modular este proceso de especiación alopátrica favoreciendo o limitando el flujo génico entre localidades lejanas. Las especies que se reproducen exclusivamente por ascosporas (ej. *P. carporrhizans*) ven favorecido el flujo génico por la gran capacidad de dispersión de las mismas, resultando en poblaciones panmícticas, de baja estructuración poblacional y menor

probabilidad de especiación. Mientras que especies isidiadas como *P. tiliácea* o las especies sorediadas como *Lobaria pulmonaria* muestran menor dispersión, mayor estructura poblacional y la posibilidad de propagación clonal rápida del entorno; estos comportamientos pueden conducir a especiación, como en la especie isidiada *Punctelia guanchica*.

Evidencias más o menos indirectas nos inducen a no descartar fenómenos simpátricos en la especiación de líquenes. A este respecto tenemos el ejemplo del género *Parmelina* en el que sugerimos que la adaptación a diferentes hábitats ha conducido a diferencias en las estrategias reproductivas en las especies *P. carporrhizans*-*P. quercina* cuyas áreas de distribución son parcialmente solapantes (Capítulo 3). También en el mismo género existen especies parafiléticas sin aislamiento geográfico *P. carporrhizans–P. atricha* (Clerc & Truong 2008), *P. tiliácea-pastillifera* (Núñez-Zapata *et al.* 2015). Y en el Capítulo 5 hallamos tres subpoblaciones genéticas en *P. carporrhizans* sin estructura geográfica; por el momento no tenemos ninguna explicación sólida de porqué se han podido generar; podría especularse que se deban a alguna adaptación al microhábitat.

A continuación, evaluaremos el avance que significa este trabajo en la consecución de los cinco objetivos específicos propuestos en la introducción:

- Hemos constatado que *Punctelia rudecta* s. lat. era un taxón polifilético, en ella reconocemos una especie norteamericana (*P. rudecta s. stricto*), una especie canaria que hemos descrito como nueva (*P. guanchica*), una sudafricana (*P. toxodes*) y otra que se distribuye en las zonas monzónicas del este de África y el Oeste de Asia que hemos “recuperado” de la literatura, recombinándola como combinación nueva (*P. ruderata*). Las muestras chilenas de *P. aff. rudecta* no sabemos bien a que corresponden, solo podemos asegurar que no pertenecen al clado norteamericano de *P. rudecta* s. stricto. Los resultados fueron apoyados por distintos análisis moleculares de coalescencia y complementariamente caracteres no moleculares como los geográficos. Además hemos generado una filogenia del género *Punctelia* con más marcadores y más muestras que las que se habían construido previamente (Crespo *et al.* 2004; Lendemer & Hodkinson 2010). Nuestra aproximación revela cinco clados principales con respaldo estadístico suficiente y que se asocia con un patrón consistente en relación con las sustancias liquénicas medulares.

- La diversidad intratalina de *Trebouxia decolorans* en *Anaptychia ciliaris* y *Xanthoria parietina*, tras el análisis realizado con microsatélites, podemos evaluarla como elevada y muy amplia o extendida en los talos analizados de estas especies. Teniendo en cuenta los resultados de estudios previos pioneros que detectaron múltiples fotobiontes en talos de líquenes con marcadores basados en secuencias de ADN (Casano *et al.* 2011) microsatélites (Mansournia *et al.* 2012) y SSCP (Muggia *et al.* 2013) sugerimos que la diversidad interna de fotobiontes Trebouxiodes es más común en los líquenes de lo que pensábamos. Además a partir de nuestros resultados discutimos las diferencias de las poblaciones de fotobiontes en las especies que hemos analizado. Así nuestro trabajo apunta a que el biotipo folioso y la alta capacidad regenerativa de *X. parietina* favorece la captación de nuevos fotobiontes y la longevidad de *A. ciliaris* favorece la acumulación de mutaciones somáticas que generan nuevos linajes clonales.

- El cultivo en condiciones aposimbióticas de micobiontes de *Parmelina carporrhizans* y *Parmelina quercina* nos ha mostrado un patrón de eyección de esporas como ascos completos con el saco ascal intacto, similar a otros líquenes (Molina & Crespo, 2000; Sangvichien *et al*. 2011; Molina *et al.* 2013). Hemos obtenido valores de eyección de esporas de los más altos en Parmeliáceos, y reportamos gran diferencia en porcentaje de germinación entre las dos especies que hemos cultivado. Además, hemos observado la secreción de compuestos fenólicos por parte del micobionte, que revelaron ser diferentes de los producidos por el talo en estado natural al ser analizados por HPLC (ver apéndice).

- Hemos desarrollado nuevos marcadores genéticos para el estudio poblacional a partir de la secuenciación del genoma del micobionte de *Parmelina carporrhizans* cultivado por nosotros aposimbióticamente. Estos marcadores son altamente específicos y variables y han sido empleados con éxito en el capítulo quinto.

- El estudio de la diversidad genética de las poblaciones de *P. carporrhizans* ha corroborado la hipótesis de que los líquenes de reproducción sexual obligada son altamente variables, y tienen menor estructuración poblacional que aquellos que son facultativamente asexuales (Werth 2010). Nuestros resultados muestran, junto con la baja estructuración, una total ausencia de clonalidad. Además hemos medido el flujo génico en forma de migración y la distribución de idiotipos sexuales del locus MAT constatando la práctica panmixia de las poblaciones de la especie. En términos generales la diversidad hallada en las poblaciones de *P. carporrhizans* responde a un modelo ecológico de núcleo-periferia donde las poblaciones en el núcleo de

distribución que se encuentra en el óptimo ecológico son más variables que las poblaciones cercanas al límite de distribución que son además dependientes de migración y sensibles a la extinción local.

CONCLUSIONES GENERALES

Altos niveles de diversidad permanecen ocultos en líquenes, enmascarados como especies crípticas, variabilidad poblacional o variabilidad intratalina.

– Hemos demostrado la polifilia de *Punctelia rudecta* describiendo una nueva especie, *Punctelia guanchica* endémica de Canarias.

– Hemos mostrado como implementar una variedad de métodos de delimitación de especies y evaluando en conjunto con datos de otro tipo como geográficos juega un papel importante en la identificación de especies.

– Hemos demostrado la ocurrencia múltiple de fotobiontes en el seno del talo liquénico, sugiriendo que la ocurrencia múltiple de fotobiontes pueda ser la norma general.

– La variabilidad de fotobionte puede ser generada internamente por mutación somática, y dependiendo de la especie, nuevos linajes de fotobiontes pueden ser captados desde el exterior.

– *Parmelina carporrhizans* y *Parmelina quercina* han mostrado diferencias en sus rasgos reproductivos en cultivo, siendo una evidencia de posibles comportamientos reproductivos diferenciales en la naturaleza debido a la adaptación a hábitats diferentes.

- Hemos secuenciado el genoma completo de *Parmelina carporrhizans* a partir de cultivo axénico, usando secuenciación de última generación (Illumina platform).

– Los marcadores microsatélites diseñados a partir del genoma de *P. caprorrhizans* son aptos para el estudio de diversidad a nivel de poblaciones genéticas.

– El estudio poblacional de *Parmelina carphorrizans* nos ha revelado gran diversidad genética y gran conectividad a lo largo de la Región Mediterránea. Hemos detectado ambos idiomorfos de los genes del locus MAT en todas las localidades.

– No hemos hallado evidencia de estructura poblacional sugiriendo que las poblaciones de P. carporrhizans actúan como componentes de una metapoblación altamente conectada por flujo génico.

– *Parmelina carporrhizans* representa un Sistema ideal para el estudio de líquenes de fertilización cruzada y reproducción sexual obligada en estudios futuros.

– La composición genética de *Parmelina carporrhizans* puede ser explicada como un modelo poblacional núcleo-periferia con gran tasa migratoria de la Cuenca Mediterránea hacia las Islas Canarias.

General conclusions

In the present thesis we have found that high levels of diversity remain hidden in lichen co-symbionts both in algae and fungi, masked as cryptic species, population diversity or intrathalline variability.

– We have demonstrated the polyphyly of *Punctelia rudecta* and described a new species, *Punctelia guanchica* endemic to the Canary Islands, additionally we epitypified.

– We showed that implementing a variety of species delimitation methods, coupled with assessment of other data types, as geography, could play an important role in identification of species and speciation process in lichenized fungi.

– We demonstrated the multiple occurrences and high photobionts diversity within the lichen thallus, suggesting that symbiont promiscuity is a general feature.

– Photobiont variability can be generated internally by somatic mutation, or depending on the species, additionally it can be acquired from the outside.

– *Parmelina carporrhizans* and *Parmelina quercina* showed differences in reproductive trade-offs in laboratory conditions suggesting differences in reproductive behavior in natural environment as adaptation to their respective habitats.

- We sequenced whole genome of *Parmelina carporrhizans* from axenic mycobiont culture, using Next Generation Sequencing (Illumina platform).

– We developed the microsatellite markers from whole genome sequence of *Parmelina carporrhizans* and showed their suitability to study population-level diversity of this lichen forming fungus.

– We showed that the strictly sexual, heterothallic lichen-forming fungus *P. carphorrizans* harbors high genetic diversity and connectivity across the Mediterranean region. We detected both mating-type idiomorphs in all populations.

– We did not find evidence for population structure, suggesting that populations of *P. carphorrizans* act as components of a metapopulation system highly connected by gene flow.

– The genetic composition of *P. carporrhizans* can thus be explained by a core-edge population model with high migration rates from the Mediterranean Basin towards the Macaronesian Islands.

– *Parmelina carporrhizans* represents an ideal system for strictly outcrossing lichen-forming fungi in further studies.

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Producción de fenoles del micobionte de *Parmelina carporrhizans* en condiciones de cultivo aposimbioticas.

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Introduccion

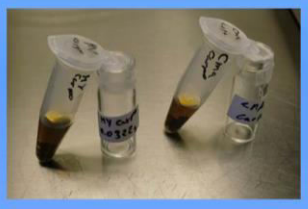
Parmelina carphorrizans , reevaluada como una especie diferente de *P. quercina* en base al estudio morfológico y molecular (Arguello et al., 2007) produce en la naturaleza:

- atranorina en el córtex
- y ácido lecanórico en la médula.

Ambos fenoles tienen aplicaciones farmacológicas en estudio, y también aplicaciones tradicionales en la industria textil y tintorera (Luo et al., 2009; Barreto et al., 2013). El cultivo de este parmelioide *in vitro*, puede resultar interesante en la producción contralada de estos compuestos fenólicos. Sin embargo los quimiosíndromes producidos por el micobionte en condiciones naturales y aposimbióticas, pueden variar ignificativamente (p. e. Molina et al., 2003):

- debido a factores bióticos (presencia o ausencia del fotobionte)
- y abióticos (nutrientes del medio, estrés hídrico, intensidad de luz, etc.)

Produccion de compuestos fenólicos en cultivo aposimbiótico



Extracción en acetona de los compuestos fenólicos.

Objetivos

- 1) Establecer las diferencias en la composición química entre el micobionte de *P. carporrhizans* en condiciones naturales e *in vitro*
- 2) determinar el interés potencial o real de los nuevos compuestos químicos que pudieran sintetizarse en cultivo.

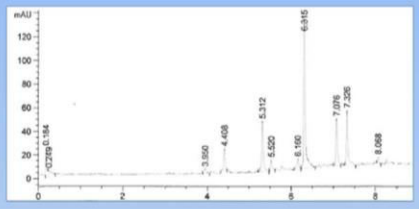
Resultados

Los talos de *P. carporrhizans* presentaron

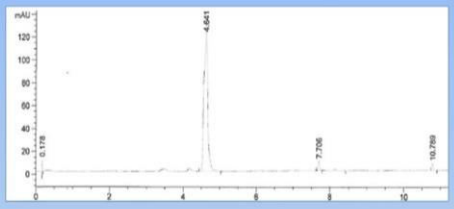
- ácido lecanórico como componente principal,
- atranorina como fenol secundario
- y trazas de cloratranorina.

Los extractos acetónicos del cultivo en condiciones axénicas mostraron un quimiosindron diferente con al menos siete compuestos aromáticos, de los cuales se identificaron tres:

- ácido confluéntico,
- ácido descarboxianziaico
- y ácido estenospórico.

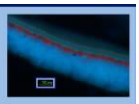


Compuestos fenólicos de cultivo de *P.carporrhizans* a: ácido confluéntico b: ácido decarboxianziaico c: ácido estenospórico.



Compuestos fenólicos del talo de *P.carporrhizans* en condiciones naturales d: ácido lecanórico e: atranorina f: cloratranorina.

A shy glance to *Parmelina carporrhizans* photobiont



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Introduction

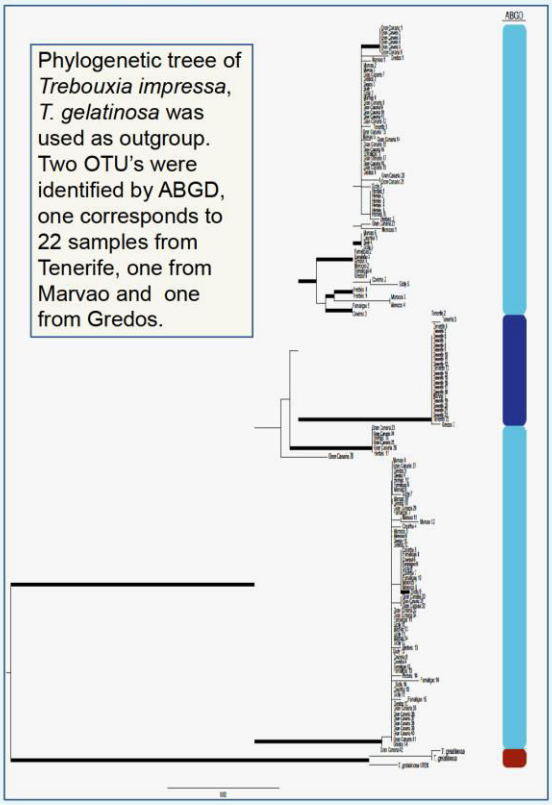
Parmelina is a small genus belonging to *Parmeliaceae* wich showed the highest photobiont specifity out of 10 *Parmeliaceae* genera (Leavitt et al. 2015). *P. carporrhizans* is a obligate sexually reproducing lichen-forming fungus which needs relichenize every generation after spore germination.

Material and methods

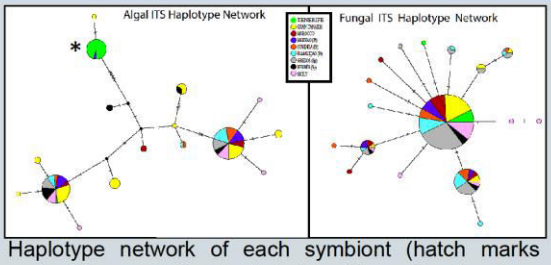
155 *P. carporrhizans* samples were collected from 9 localities: Canary Islands (2), North Africa (1), Iberian Peninsula (5) and Sicily (1).
ITS region was sequenced for both, mycobiont and photobiont.
Haplotype networks were constructed for both bionts with Pop Art 1.7
For photobiont Maximum Likelihood tree was performed with RaxML and OTUs were delimited with Automated Barcode Gap Discovery (Puillandre et al. 2012).
Molecular index were calculated with Arlequin 3.1.

Results

Phylogenetic treee of *Trebouxia impressa*, *T. gelatinosa* was used as outgroup. Two OTU's were identified by ABGD, one corresponds to 22 samples from Tenerife, one from Marvao and one from Gredos.



The photobiont variability (23 parsimony informative sites) resulted higher than mycobiont (6 parsimony informative sites) in *P. carporrhizans*



Haplotype network of each symbiont (hatch marks represents mutations). Asterisk (*) marks the less related haplotype

Molecular Diversity	GranCanaria	Tenerife	Morocco	Famaliçao	Herbés	Gredos	Sicily	Marvao	Covinha
Sample Size	42	23	8	15	14	14	15	14	10
Number of Alleles	8	2	4	5	4	4	8	6	3
transitions	25	9	18	17	25	18	15	20	16
transversions	11	4	8	8	10	12	7	11	9
Heterozygosity	0.327	0.027	0.338	0.275	0.310	0.332	0.331	0.347	0.265
Theta_pi	13.748	1.130	13.643	11.086	13.011	13.451	9.333	13.945	11.111

	GranCanaria	Tenerife	Morocco	Marvao	Covinha	Famaliçao	Gredos	Herbés	Sicily
Allele 1	12	0	4	6	7	10	8	3	6
Allele 2	6	0	0	0	0	0	0	0	0
Allele 3	1	0	0	0	0	0	0	0	0
Allele 4	2	0	0	0	0	0	0	0	0
Allele 5	1	0	0	0	0	0	0	0	0
Allele 6	4	0	0	0	0	0	0	2	0
Allele 7	13	1	0	4	0	1	3	7	2
Allele 8	3	0	0	0	0	0	0	0	0
Allele 9	0	22	0	1	0	0	1	0	0
Allele 10	0	0	1	0	0	0	0	0	0
Allele 11	0	0	2	0	0	0	0	0	0
Allele 12	0	0	1	1	2	1	2	0	2
Allele 13	0	0	0	0	0	2	0	0	0
Allele 14	0	0	0	0	1	1	0	0	0
Allele 15	0	0	0	0	0	0	0	2	0
Allele 16	0	0	0	0	0	0	0	0	1
Allele 17	0	0	0	0	0	0	0	0	1
Allele 18	0	0	0	0	0	0	0	0	1
Allele 19	0	0	0	0	0	0	0	0	1
Allele 20	0	0	0	0	0	0	0	0	1
Allele 21	0	0	0	1	0	0	0	0	0
Allele 22	0	0	0	1	0	0	0	0	0

The number of haplotypes and the variability observed in Tenerife is the lowest of the 9 sites.
Allele 1 was found in all populations except Tenerife.

Discussion

- Our results of *P. carporrhizans* photobiont are in acordance with the specificity reported for the genus *Parmelina* (Leavitt et al. 2015) and the photobiont distribution pattern of *Ramalina farinacea* (del Campo et al. 2010).

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Picture from: Francesco Dal Grande